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IDENTIFICATION AND CHARACTERIZATION OF  
A NOVEL CLASS OF PHARMACOLOGIC AGENTS  
FOR THE REVERSAL OF MULTIDRUG RESISTANCE

JAMES MATTHEW FORD

1999



YALE



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**IDENTIFICATION AND CHARACTERIZATION OF  
A NOVEL CLASS OF PHARMACOLOGIC AGENTS  
FOR THE REVERSAL OF MULTIDRUG RESISTANCE**

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Medicine

by  
James Matthew Ford  
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## ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL CLASS OF PHARMACOLOGIC AGENTS FOR THE REVERSAL OF MULTIDRUG RESISTANCE. James M. Ford and William N. Hait. Section of Medical Oncology, Departments of Internal Medicine and Pharmacology, Yale University School of Medicine, New Haven, CT.

Phenothiazines and structurally related compounds inhibit cellular proliferation and sensitize multidrug resistant (MDR) cells to chemotherapeutic agents. To identify more potent pharmaceuticals, the structure-activity relationships of a series of phenothiazines and related compounds were studied in MDR MCF-7/DOX human breast cancer cells. Spectrophotometric microtiter assays were used to determine the antiproliferative and anti-MDR effects of drugs alone or in combination with chemotherapeutic agents. Substitutions on the phenothiazine ring that increased hydrophobicity increased antiproliferative and anti-MDR activities. For example, -Cl and -CF<sub>3</sub> groups increased whereas -OH groups decreased potency. Modifying the length of the alkyl bridge (4 C > 3 or 2) and the type of amino side chain (piperazinyl > non-cyclic) also influenced potency. Compounds with tertiary amines were better anti-MDR agents than those with secondary or primary amines, but were equipotent antiproliferative agents. The effects of these substituents were unrelated to hydrophobicity. The structure-activity relationships suggest that an ideal structure for reversing MDR by phenothiazines has a hydrophobic nucleus with a -CF<sub>3</sub> ring substitution at position 2, connected by a 4 carbon alkyl bridge to a para-methyl substituted piperazinyl amine. Related compounds having certain of these properties were studied and the thioxanthene class of antipsychotics were identified as having increased activity and potency as chemosensitizers. For example, *trans*-flupenthixol, the most effective of these compounds, decreased doxorubicin, vinblastine and colchicine resistance, respectively, by 15-, 36-, and 8-fold in MCF7/DOX cells; by 35-, 40-, and 20-fold in the MDR human





carcinoma line KB-V1; and by 7-fold (doxorubicin) and 4-fold (vinblastine) in the murine leukemia line P388/DOX. *Trans*-flupenthixol fully reversed 20-fold doxorubicin and 100-fold colchicine resistance in an NIH 3T3 fibroblast line transfected with an expression vector containing the *mdr1* gene. Equimolar concentrations of *trans*-flupenthixol caused 1.5- to 2-fold greater antagonism of MDR than its stereoisomer *cis*-flupenthixol, 1- to 3-fold greater than the calcium channel blocker verapamil, and 2- to 12-fold greater than the phenothiazine homolog fluphenazine. None of these agents altered drug resistance or cross-resistance in a non-P-glycoprotein expressing MCF-7 cell line selected with mitoxantrone, nor any of the parental drug-sensitive cell lines. *Trans*-flupenthixol was not accumulated more than *cis*-flupenthixol in MDR cells, implying that their stereospecific anti-MDR effects were not the result of selective differences in the access of the drugs to intracellular targets. Both drugs caused increased accumulation of doxorubicin in MDR cells, but not in sensitive cells, suggesting that they modulate MDR by interacting with a uniquely overexpressed cellular target in these resistant cells. The *cis*- and *trans*-stereoisomers were equally active antagonists of protein kinase C and calmodulin. The apparent lack of clinical toxicity of *trans*-flupenthixol makes it an attractive drug for further investigation, and a pilot *in vivo* trial was performed in a murine tumor model, which showed *trans*-flupenthixol caused increased doxorubicin accumulation in MDR ascites cells *in vivo*, but was toxic to mice at the doses tested. This thesis shows that *trans*-flupenthixol, a drug that lacks extrapyramidal side effects in humans, is a potent, stereospecific antagonist of MDR in cells that overexpress P-gp.



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## DEDICATION

To my wife, Diane, for her love, understanding and encouragement throughout our many years together.



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## LIST OF ABBREVIATIONS

ANLL	Acute Nonlymphoblastic Leukemia
CNS	Central Nervous System
Ca <sup>++</sup>	Calcium
CaM	Calmodulin
CML	Chronic Myelogenous Leukemia
CsA	Cyclosporin A
DMDP	<i>N</i> -(3,4-dimethoxyphenethyl)- <i>N</i> -methyl-2-(2-naphthyl- <i>m</i> -dithane) 2-propylamine
GSH	Glutathione
GST	Glutathione- <i>S</i> -Transferase
ILS	Increased Life Span
i.p.	Intraperitoneal
MDR	Multidrug Resistance
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl Tetrazolium Bromide
NASAV	<i>N</i> -( <i>p</i> -azidosalicyl) aminomethyl verapamil
NASV	<i>N</i> -( <i>p</i> -azidosalicyl)- <i>N'</i> -(β-aminoethyl) vindesine
P-gp	P-glycoprotein
PKC	Protein Kinase C
PTZ	Phenothiazine
TPA	12- <i>O</i> -tetradecanoyl phorbol-12-acetate
W5	<i>N</i> -(6-aminoethyl)-1-chloronaphthalensulfonamide
W7	<i>N</i> -(4-aminoethyl)-5-chloronaphthalensulfonamide
W12	<i>N</i> -(6-aminobutyl)-2-naphthalensulfonamide
W13	<i>N</i> -(6-aminobutyl)-5-chloronaphthalensulfonamide





## Chapter I

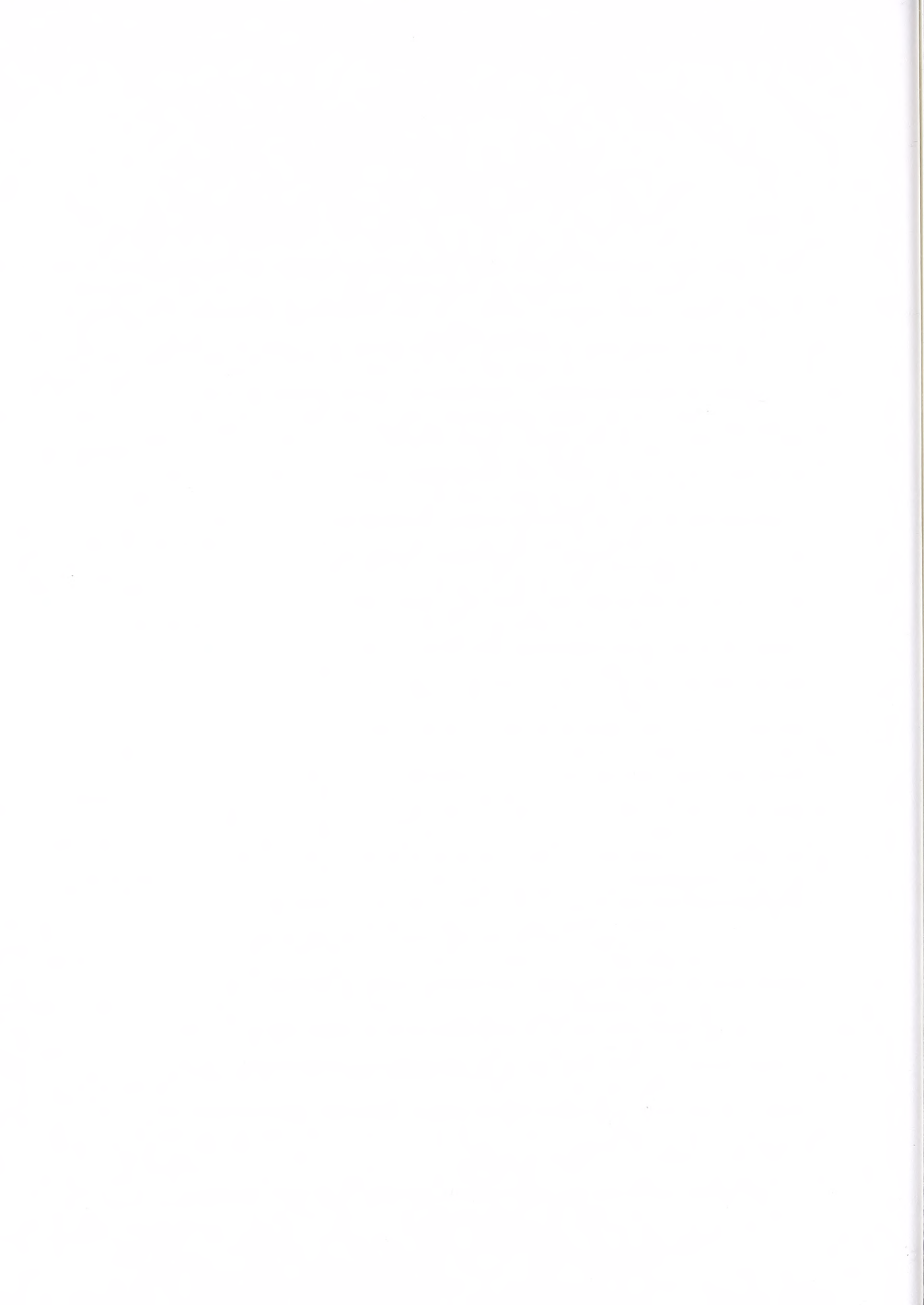
### INTRODUCTION

#### A. Overview of the Cellular and Molecular Biology of Multidrug Resistance

##### 1. *Definition and Characteristics of the Multidrug Resistant Phenotype*

Tumor cell resistance to chemotherapeutic drugs is considered to be a major problem in the clinical treatment of cancer. One form of drug resistance, termed multidrug resistance (MDR), is defined as the ability of cells exposed to a single cytotoxic agent to develop resistance to a broad range of structurally and functionally unrelated drugs (24). Experimental models of MDR have been obtained by growing cultured human, murine or hamster neoplastic cell lines in progressively increasing concentrations of cytotoxic drugs, although MDR transplantable tumors have been developed *in vivo* (48). Cells selected for resistance to one drug display significant cross-resistance to the other drugs within the MDR phenotype, which mainly include the "natural product" anti-cancer drugs such as the anthracyclines, the *Vinca* alkaloids, the epipodophyllotoxins, colchicine, and actinomycin D, but not drugs such as bleomycin, methotrexate or alkylating agents. The level of cross-resistance displayed by MDR cells to individual drugs varies between cell lines. However, the qualitative similarity in the pattern of drug resistance suggests a single underlying mechanism is responsible for MDR. Since many of the drugs affected by MDR are believed to possess different mechanisms of cellular toxicity, investigations into the determinants of MDR have focused on the identification of a unifying cellular defense mechanism against toxic agents rather than on individual alterations in target enzymes. The most consistent changes found in MDR cell lines are an increased expression of a high molecular weight cell surface glycoprotein (P-glycoprotein) and a decreased accumulation and retention of cytotoxic drugs (186).

Several excellent and thorough reviews of MDR have been recently published (14, 27,



168). Thus, a brief summary of the current understanding of the cellular pharmacology and molecular biology of this form of drug resistance will be presented. Following this will be a detailed discussion of the pharmacological reversal of MDR, since this field of research has not been adequately reviewed.

## ***2. Cellular Pharmacology of Multidrug Resistance***

The first experiments demonstrating that MDR cells were associated with a relative decrease in intracellular cytotoxic drug accumulation when compared to sensitive, parental cells were performed independently by Victor Ling and June Biedler, using Chinese hamster ovary cells resistant to colchicine and daunomycin, respectively (138, 184). Subsequently, every well-characterized MDR cell line has been shown to display decreased drug accumulation, generally ascribed to enhanced efflux rather than diminished influx (107, 108). A number of investigators explored the basis for this decreased drug retention, and found an energy-dependent mechanism responsible for the enhanced efflux. Accordingly, experiments in which MDR cells were depleted of ATP-energy by removing glucose or adding metabolic inhibitors caused a reversal of the accumulation defect, while replacing glucose restored MDR (49). In contrast, anthracycline drug influx appeared to occur by simple passive diffusion, and to be similar in sensitive and MDR cells (128). These and other observations led to the acceptance of the still unproven hypothesis that an active efflux pump of broad specificity is responsible for the transport defect in MDR cells and results in altered sensitivity to multiple drugs (212).

It is important to note that alternative explanations have been advanced to explain decreased efflux, including alterations in the binding of drugs to cellular proteins or organelles, particularly for colchicine (14). The determinants of altered drug transport in MDR cells are complex and remain to be fully defined. However, the drug efflux pump hypothesis has remained consistent with recent advances in the understanding of the MDR phenotype.



### *3. Molecular Biology of Multidrug Resistance*

#### *3a. Overexpression of P-glycoprotein*

An approximately 170 kDa plasma membrane glycoprotein associated with MDR cells, but not detectable in drug-sensitive cells, was first identified by Ling's group (121), and found to correlate with both decreased drug accumulation and degree of resistance in Chinese hamster ovary cells (125). Since then, several monoclonal antibodies to P-glycoprotein (P-gp) have been developed, and nearly every established MDR cell line from mouse, hamster, or human origin has been shown to express a 150 - 180 kDa membrane protein immunologically cross-reactive with the originally described P-gp (124, 125, 126).

A great deal of evidence has accumulated suggesting that P-gp may be the active efflux pump of broad specificity as predicted by studies of the cellular pharmacology of MDR. For instance, purified, solubilized P-gp has been shown to possess an ATPase function (93). A number of radiolabelled drugs, such as [<sup>3</sup>H]-vinblastine, and photoactivatable drug analogs, have been shown to bind in a specific, saturable manner to MDR plasma membrane vesicles, but not to sensitive cell vesicles (43, 45). Furthermore, the major labelled membrane protein has a molecular weight of 170 kDa and is immuno-cross-reactive with monoclonal antibodies to P-gp (43, 44, 198). Structural analyses of P-gp accomplished by sequencing cDNA clones from P-gp encoding genes are particularly provocative (38, 82). The deduced amino acid sequence of P-gp reveals it to be dimer consisting of 6 transmembrane segments, and bearing striking homology to the ATP-binding domains of a group of multicomponent bacterial periplasmic transport proteins, including the bacterial membrane  $\alpha$ -hemolysin export pump (hylB) (100). Taken together, this experimental evidence strongly suggests that expression of P-gp forms the genetic basis for MDR. However, whether the protein binds and exudes drugs by an active transport process, physically opens channels in membranes through which drugs pass, or even irreversibly binds drugs and is removed altogether from the cell is not known.





### 3b. Overexpression of *mdr* Genes

In certain MDR cell lines, such as the human epidermal KB lines, the amplification of gene(s) coding for P-gp allowed the use of in-gel renaturation (187) to isolate and ultimately clone *mdr* genes from hamster, mouse and human cell lines (83, 188). Cloned gene segments from the human KB cells (*mdr1* gene) hybridized to a 4.5 kb mRNA present in MDR cells in proportion to the degree of drug resistance, to which complementary cDNAs were prepared (38). Alternatively, monoclonal antibodies to P-gp were used as probes to screen a cDNA expression library for P-gp encoding clones from a Chinese hamster ovary MDR cell line (76). The cDNA clones obtained by these two methods were found to be highly homologous and to hybridize strongly to each other, indicating that P-gp is the product of the *mdr1* gene (236). Sequencing these cDNAs revealed the structural characteristics of P-gp discussed above.

Perhaps the most compelling evidence demonstrating that MDR is due to the expression of a single gene came from transfection experiments. Transfer of total genomic DNA from MDR human cells (207), and more recently transfection and expression of retroviral vectors containing full length cDNAs coding for P-gp (235) into sensitive cells conferred the full MDR phenotype in the wild-type cells.

## B. Clinical Relevance of Multidrug Resistance

An important question posed by the tremendous advances made in understanding the cellular and molecular biology of MDR is whether the expression of the *mdr1* gene product and the MDR phenotype occur in human tumors and are responsible for intrinsic or acquired anti-cancer drug resistance. Most experimentally induced MDR cell lines have been selected for 100-fold or greater levels of drug resistance, whereas clinical drug resistance is generally thought to be on the order of 2- to 5-fold (23). Therefore, it is possible that the pharmacologic characteristics displayed by highly MDR cell lines are a laboratory phenomenon only, exploiting otherwise functionally unrelated cellular mechanisms, and



bearing no relevance to clinical drug resistance. Nevertheless, the common observation that patients who relapse from therapy with certain drugs often become refractory to treatment with virtually all drugs is particularly suggestive of the MDR phenotype, and demands careful exploration.

### *1. Expression of P-glycoprotein in Normal Human Tissues*

Critical to the understanding of the clinical importance of MDR is the determination of the normal physiologic role and tissue distribution of P-gp, particularly as a baseline to which tumor expression may be compared. A variety of normal human tissues samples have been studied for P-gp expression using immunohistochemical or immunoperoxidase staining methods with monoclonal antibodies against P-gp. High levels of P-gp have been found in adult adrenal glands, kidney and placenta (218), and more abundantly in adrenal cortex than adrenal medulla (219). At a cellular level, P-gp has been localized to the luminal or apical surface of liver, pancreas, kidney, colon, and jejunum (222), suggesting a possible role in secretory processes. Immunoblot assays have also demonstrated the presence of P-gp in liver, and low levels in small bowel (27). It was recently reported that endothelial cells of human capillary blood vessels at the blood-brain barrier and blood-testis barrier also express P-gp, but cells from larger blood vessels or other tissue capillaries do not, suggesting that P-gp may assist these tissues in their known function of excluding various soluble polar compounds from the CNS and other pharmacologic sanctuaries (42). Examination of P-gp expression in non-neoplastic tissues has also been carried out at the genetic level, utilizing *mdr1* gene hybridization probes and Northern or slot blot analysis. An initial survey of tissue types found low levels of *mdr1* mRNA in most samples, with higher levels in adrenal, kidney, liver and colon (65), although significant variability existed.

Based on these findings, it is reasonable to believe that P-gp possesses a normal physiologic function in human tissues, perhaps related to secretion and/or protection of tissues from various naturally occurring toxins or even commonly encountered xenobiotics



structurally related to the natural product antibiotic drugs. It is likely that the continued analysis of this problem will eventually yield a natural substrate for P-gp, since it is doubtful that evolution would have selected for and highly conserved across species a protein whose primary function is to bind or extrude chemotherapeutic drugs.

## 2. *Expression of P-glycoprotein in Human Tumors*

Identical methods to those just described have been used to analyze P-gp and *mdr1* mRNA expression in human tumor samples. Immunoblot assays have revealed overexpression of P-gp in 2 of 5 cases of advanced non-responsive ovarian cancer (18), and in sequential biopsies from 2 patients with acute nonlymphoblastic leukemia (ANLL) progressing on therapy (142). Similar findings of a relationship between P-gp expression and clinical resistance to anti-cancer therapy was reported by Carulli *et al* (31) for 2 patients with chronic myeloid leukemia (CML) in blast crisis and one patient with ANLL. Gerlach *et al* (75) reported that approximately 20% of biopsies specimens from 46 patients with 12 different types of sarcomas expressed increased levels of P-gp, though a correlation was not found between prior chemotherapy and P-gp expression.

Measurement of *mdr1* mRNA has revealed high levels of expression independent of treatment in certain tumors derived from tissues known to overexpress P-gp normally, such as pheochromocytomas (from adrenal medulla) and adrenocortical tumors (from adrenal cortex) (65). Similarly, *mdr1* mRNA expression in untreated renal cell carcinomas was higher than in other urogenital tumors derived from tissues which do not normally express P-gp (122). Interestingly, the levels of mRNA from tumors in this study were shown to correlate inversely with tumor cell vinblastine sensitivity, but not doxorubicin or cisplatin sensitivity, as determined by an *in vitro* cellular toxicity assay.

The most extensive study of *mdr1* expression reported to date analyzed over 400 tumor samples taken from patients both treated and untreated with chemotherapeutic drugs (79). Expression was found to be high in untreated colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, pheochromocytoma and CML in blast crisis, while



untreated breast cancer, non-small cell lung cancer, CML in chronic phase and many other tumor types had low or undetectable levels of *mdr1* mRNA. Expression in tumors from patients who initially responded but subsequently relapsed on chemotherapy was high in non-Hodgkin's lymphoma, neuroblastoma, pheochromocytoma, breast cancer, and acute lymphocytic leukemia. Therefore, P-gp overexpression was observed independent of treatment in a number of intrinsically resistant tumors, and seen to increase in frequency in several tumor types which received treatment. However, to prove an association between intrinsic or acquired drug resistance and P-glycoprotein expression, pre- and post-treatment P-gp levels from individual patients must be determined, and careful analyses of the possible correlations between P-gp levels and treatment responses performed.

These important initial studies have proven that P-gp is expressed in both normal and neoplastic human tissues. Whether P-gp expression is a marker for intrinsic drug resistance; whether its increased expression is related to the development of acquired resistance; or what its functional role is in normal tissues, are all questions which will be the focus of future studies. In addition, this work demonstrates that immunological and molecular diagnostic techniques may be successfully used to detect P-gp and *mdr1* gene expression in clinical samples, and suggests their use for identifying individual patients who may be appropriate candidates for trials designed to circumvent or modify MDR in humans.

### C. Alternative Mechanisms Involved in Multiple Drug Resistance

While the study of MDR has focused most extensively on P-gp associated experimental models, a rapidly increasing number of biochemical and molecular alterations have been recently described in cell lines selected for the expression of resistance to multiple drugs. Certain of these changes appear to occur only in consort with overexpression of the *mdr1* gene, suggesting they may be the product of genes passively transcribed along with *mdr1* amplification. In fact, these type of findings have encouraged investigators to thoroughly characterize and define MDR cell lines as "classic" P-gp associated cells with drug





accumulation defects, or otherwise. The two best characterized alternative mechanisms for a broad multiple drug resistance are changes in the expression of glutathione drug detoxification enzymes, and changes in topoisomerase II.

The multifunctional phase II detoxification enzyme glutathione-*S*-transferase (GST) catalyzes the conjugation of electrophilic substances and endogenous xenobiotics to the tripeptide thiol, glutathione (GSH), forming stable, excretable metabolites, and prevents oxidative damage through intrinsic, organic peroxidase activity (36, 115). GST has been found to be increased in several cell lines associated with resistance to individual alkylating agents (220). However, an anionic ( $\pi$ ) isozyme of GST has recently been found to be greatly overexpressed in P-gp positive MCF-7/DOX cells selected for MDR with doxorubicin (12). Transfection and expression of cDNA clones of this isozyme in drug sensitive MCF-7 cells conferred low level (1.5- to 3-fold) resistance to alkylating agents, but no increase in resistance to natural product drugs (151). Thus, GST's role in multiple drug resistance involving non-alkylating agents has not yet been shown. Other evidence, however, does suggest that the GSH system may have some role in 'non-P-gp' multiple drug resistance. For instance, depletion of MCF-7/DOX cellular GSH, the substrate for all GST enzymes, with the  $\gamma$ -glutamylcysteine synthetase inhibitor buthionine sulfoximine (147), resulted in a 5- to 7-fold increase in cellular sensitivity to doxorubicin (54).

The topoisomerases are enzymes that catalyze the breaking and rejoining of DNA required for genomic unwinding, and are necessary for DNA replication (140). Attention has focused on the role of topoisomerase II in drug resistance, since it is an enzyme target for many DNA intercalating and non-intercalating neoplastic agents such as doxorubicin, mitoxantrone, etoposide, teniposide and mAMSA (39, 156, 221). Alteration in the amount or function of topoisomerase II has been suggested as a possible mechanism for cellular resistance to this broad group of drugs (190), and in fact, several multiple drug resistant cell lines characterized by decreased levels of topoisomerase II activity in the absence of P-gp expression or accumulation defects have now been reported (15, 35, 171).

A great number of other changes in cells resistant to multiple drugs have also been



described. For instance, several low molecular weight cytosolic proteins are uniquely expressed in MDR, such as the  $\text{Ca}^{++}$ -binding protein sorcin (148), and others (59). Similarly, several membrane proteins unique to MDR cells have been observed in addition to P-gp, such as an 85 kDa glycoprotein in K562/ADM cells (91), and a 150 kDa protein immunologically distinct from P-gp (146). Whether these membrane proteins are metabolic products of P-gp degradation has not been adequately studied.

Many biochemical changes have been noted in MDR cells, particularly in MCF-7/DOX cells, including changes in phase I cytochrome P450 enzymes like aryl hydrocarbon anhydroxylase (114), alterations in the regulation of the hexose monophosphate shunt (244), and increased activity of the drug metabolizing enzymes DT-diaphorase and glucuronyl transferase (46).

It is unclear for most of these changes whether their presence or alteration in MDR cells have a significant regulatory or functional role, or are simply a coincidental result of the selecting procedure, resulting from co-amplified genes related to P-gp expression by their physical proximity to the *mdr1* gene, but having no other functional or mechanistic involvement in the MDR phenotype. Thus, numerous opportunities await researchers interested in further defining the regulatory mechanisms for "classic" MDR, and in exploring the molecular and biochemical basis for alternative forms of multiple drug resistance.

## **D. Pharmacologic Reversal of Multidrug Resistance by Chemosensitizers**

### **1. Introduction**

A major goal in experimental as well as clinical investigations of any form of drug resistance is to devise or discover unique methods by which to successfully reverse or antagonize this resistance. Great interest, therefore, has been recently focused on the pharmacologic reversal of MDR, especially in *in vitro* models. Through the identification of specific compounds or classes of agents which reverse MDR, investigators hope to gain a



better understanding of the various biochemical mechanisms involved in this form of cellular drug resistance, and to provide possible agents for use in clinical trials.

The first report of the pharmacologic reversal of MDR came from Takashi Tsuruo and his colleagues, who showed that the calcium channel blocker verapamil greatly potentiated the antiproliferative activity of vincristine, in association with an increased cellular accumulation of vincristine, in a MDR murine leukemia cell line *in vitro* and *in vivo* (228). Since this original observation, a large number of compounds have been shown to have activity in a variety of cell lines and in *in vivo* tumor models when co-administered with cytotoxics, for at least partially reversing MDR. Tables 1-1 through 1-6 summarize the pharmacologic agents, MDR cells lines, and cytotoxic drugs used to date, both *in vitro* and *in vivo*, for demonstrating reversal of MDR. It must be noted that a variety of assays have been used by various investigators to measure the antiproliferative or cytotoxic effects of drugs, resulting in a broad range of reported values for similar agents. In general, these chemical compounds, termed "chemosensitizers" or "resistance modifiers," act to alter the drug accumulation defect present in MDR cells, and cause little or no potentiation of drug cytotoxicity in wild-type, or intrinsically sensitive cells. While there are exceptions, investigators generally determine the magnitude of chemosensitizers' effects by comparing the doses necessary to inhibit cell growth by 50% ( $IC_{50}$ ) for a cytotoxic drug to which cells are resistant in the absence versus presence of a relatively non-toxic, fixed concentration of chemosensitizer. The ratio of these two values is referred to by different investigators as the the fold-sensitization, Dose Modifying Factor, Degree of Potentiation, or MDR Ratio. This standard has been used to calculate the fold reversal of cellular drug resistance for each chemosensitizer listed in Table 1-1 to 1-6.

Very little is yet known regarding the biochemical mechanism, or even the specific molecular targets, for any of the structurally diverse group of identified chemosensitizers (27), partly because many of these compounds have profoundly different effects on cellular physiology, often including significant cytotoxicity of their own. Nevertheless, the results from this field of study have generated considerable interest in the possibility of devising



clinical protocols utilizing relatively non-toxic chemosensitizers to circumvent clinical drug resistance in humans (149, 165, 214).

The chemosensitizers described to date may be grouped into five broad categories: (a) calcium channel blockers, (b) calmodulin antagonists, (c) non-cytotoxic anthracycline and *Vinca* alkaloid analogs, (d) otherwise unrelated hydrophobic, cationic compounds, and (e) cyclosporines. Though this diverse group of compounds share only broad structural similarities, all are extremely lipophilic, and those in the first four groups are all heterocyclic, amphipathic substances. This suggests that there may be one or more specific receptor sites for anti-MDR drugs, which have unique, though as yet poorly defined, structural requirements for efficient binding.

The following sections will review the literature to date regarding the effects of chemosensitizers *in vitro*, *in vivo*, and in human trials, and will focus on the need to carefully define the structural requirements for antagonism of MDR by chemosensitizers and to elucidate their mechanism of action.

## 2. *In Vitro Effects of Chemosensitizing Agents*

### 2a. *Verapamil*

Based on the knowledge that certain MDR cell lines displayed an increase in drug efflux due to alterations in drug transport at the plasma membrane level (107), Tsuruo's group examined the anti-MDR effect of the calcium channel blocker verapamil, presumably because of its already known action on a membrane target (62) and its documented inhibitory effect on several hormonal secretory functions (55). Using a tissue culture growth inhibition assay, it was shown that 2.2 to 6.6  $\mu\text{M}$  verapamil completely reversed the approximately 30-fold resistance to vincristine and 7-fold resistance to vinblastine displayed by a MDR murine leukemia cell line, P388/VCR (228). Incubation with 6.6  $\mu\text{M}$  verapamil for 5 hours also caused a 10-fold increase in accumulation of [ $^3\text{H}$ ]-vincristine in P388/VCR cells. In addition, verapamil caused a 3-fold increase in the antiproliferative effect of vincristine and vinblastine and a 2-fold increase in [ $^3\text{H}$ ]-vincristine accumulation in sensitive





P388 cells (228). Doses of verapamil greater than 6.6  $\mu\text{M}$  had significant cytotoxicity alone. Since verapamil was shown not to alter vincristine binding to tubulin, the apparent cytotoxic target of *Vinca* alkaloids (164), it was concluded that verapamil's pharmacologic anti-MDR effect was due to alterations in drug accumulation (228).

In subsequent reports, Tsuruo's group similarly demonstrated that 6.6  $\mu\text{M}$  verapamil fully reversed the 20-fold vincristine resistance and the 3-fold doxorubicin resistance in a MDR human acute myelogenous leukemia line K562/VCR (230), and partially reversed the 40-fold doxorubicin resistance in P388/ADM cells (229). Again, this anti-MDR effect was associated with modest (5-fold) increases in chemotherapeutic drug accumulation, shown to be due specifically to inhibition of an energy-dependent efflux mechanism (229).

Since these early observations, a large number of investigators have demonstrated the chemosensitizing activity of verapamil in various cell lines (Table 1-1) using a variety of techniques. For example, resistance to chemotherapeutic selecting agents was partially reversed by verapamil in vinblastine resistant human leukemic lymphoma CCRF-CEM cells (13), and in doxorubicin resistant B16 murine melanoma cells (66), as measured by short-term cell growth inhibition. Clonogenic assays revealed modest increases in drug cytotoxicity by 1  $\mu\text{M}$  verapamil plus daunomycin in MDR Chinese hamster ovary cells (29), and 6  $\mu\text{M}$  verapamil plus doxorubicin in doxorubicin resistant C<sub>6</sub> rat glioblastoma cells (102). Slater and colleagues (215) showed that 2 to 6  $\mu\text{M}$  verapamil caused significant increases in the inhibition of DNA and RNA synthesis by daunomycin in daunomycin resistant Ehrlich ascites carcinoma cells, as measured by [<sup>3</sup>H]-uridine and [<sup>3</sup>H]-thymidine incorporation. Several samples of fresh human tumor cells from patients who relapsed on doxorubicin were shown to be more sensitive to the drug in the presence of verapamil by the human tumor clonogenic assay (80). In addition, the sensitivity of most of the parental, wild-type cells from which the MDR lines were derived, as well as other intrinsically sensitive cell lines, was not significantly affected by verapamil at non-cytotoxic doses.

The effect of the verapamil on modifying cellular cross-resistance to chemotherapeutic



drugs other than those used for selection is of great interest, both with respect to the mechanism and pharmacology of verapamil's anti-MDR activity, and in defining the molecular mechanism of MDR itself. However, conflicting results have been reported, complicated by the fact that many of these studies occurred before the characterization of cell lines using molecular and immunologic probes for P-glycoprotein expression became common. Using a MES-SA human uterine sarcoma cell line selected for 100-fold resistance to doxorubicin and recently shown to overexpress the *mdr1* gene product (211), Harker and coworkers showed that 6  $\mu$ M verapamil caused a 7-fold enhancement of doxorubicin effect, associated with increased [ $^{14}$ C]-doxorubicin accumulation and retention (95). Verapamil caused a similar partial reversal of this cell line's significant cross-resistance to daunomycin, actinomycin D and mitoxantrone, but did not effect cell resistance to agents such as etoposide or melphalan, drugs not included in the phenotypic definition of MDR. Paradoxically, verapamil resulted in increased accumulation of [ $^3$ H]-vinblastine in these cells, but did not alter MES-SA/Dx5 resistance to vinblastine toxicity.

Conversely, Beck's group reported that 10  $\mu$ M verapamil caused a 75- and 87-fold decrease in the 244- and 1163-fold CEM/VLB<sub>100</sub> cell resistance to vinblastine and vincristine, respectively, but only a 2- to 5-fold potentiation of doxorubicin and daunomycin, to which the cells were 100-fold cross-resistant (17). Fojo *et al* (64) demonstrated that 20  $\mu$ M verapamil caused a full reversal of the 20- to 70-fold cross-resistance to doxorubicin, vinblastine and vincristine in colchicine selected KB human carcinoma cells, while causing only a 60-fold reduction in the 220-fold resistance to colchicine. Finally, in doxorubicin selected P388/ADR cells, the degree of cytotoxic potentiation for a series of five anthracyclines by 10  $\mu$ M verapamil was shown to be linearly related to the level of cross-resistance displayed to these drugs (131). Thus, while verapamil equally affects cellular resistance to selecting agents and cross-resistance to other drugs in some MDR cell lines, this finding is not true for other MDR cell lines. The question of why various MDR cells display different degrees of resistance and cross-resistance to drugs, and why some cross-resistance is refractory to modulation by verapamil



is presently unclear. An intriguing possibility is that alterations in the *mdr1* gene at the genomic level (40) or post-translational modifications in P-glycoprotein may cause changes in the affinity of certain drugs or drug classes for the putative drug-binding site(s), or may render P-glycoprotein less susceptible to antagonism by verapamil. Alternatively, multiple isoforms of P-glycoprotein, as well as multiple mechanisms of drug resistance to various chemotherapeutic agents operating within a single cell line may also explain these phenomena.

While the target and mechanisms for verapamil's pharmacologic effects on MDR cells is unclear, the effect of verapamil on the well-characterized changes in drug accumulation observed in MDR cells has been carefully documented. Kessel and Wilberding (128) probed the effect of verapamil on anthracycline cellular kinetics in P388/ADR cells. Drug influx was not altered by verapamil nor by the metabolic inhibitor sodium azide, consistent with a diffusional model of anthracycline inward transport. Drug efflux from [<sup>3</sup>H]-daunomycin loaded cells was also not affected by either modifying agent in P388 sensitive cells, while MDR cell daunomycin efflux was inhibited by varying degrees with 2 to 20  $\mu$ M verapamil, suggesting that verapamil specifically inhibits the energy-dependent drug efflux mechanism described for these cells (108). More recently, similar studies were performed with the human myeloma MDR cell line 8226/DOX40 (19). Accordingly, 10  $\mu$ M verapamil caused an increase in net [<sup>14</sup>C]-doxorubicin accumulation in resistant, but not sensitive cells, due to a decrease in drug efflux. Verapamil was shown not to alter initial anthracycline influx during the first 60 seconds of drug exposure. Furthermore, the quantitative formation of single-stranded, double-stranded and protein-associated DNA breaks in both sensitive and resistant cells after a one hour doxorubicin exposure in the absence and presence of verapamil was determined by DNA alkaline elution techniques (19), since a proposed mechanism for anthracycline cytotoxicity is through topoisomerase II mediated DNA strand breaks (189). In resistant cells there was an approximately 5-fold decrease in the formation of DNA lesions compared to sensitive cells. However, in the presence of 10  $\mu$ M verapamil, sensitive and resistant cells sustained equivalent amounts of



DNA damage by doxorubicin.

Therefore, it has been clearly shown that verapamil inhibits the energy-dependent drug efflux common to MDR cells. In addition, it is highly likely that the drug cytotoxicity potentiated by verapamil is secondary to the resultant increase in intracellular drug accumulation.

Verapamil is the best studied chemosensitizer to date, and is a potent and effective antagonist of resistance to a number of drugs in most MDR cell lines, *in vitro*. However, verapamil possesses potentially life-threatening vasodilatory effects in humans at plasma levels in the 2 to 6  $\mu\text{M}$  range (28, 50) and is cytotoxic itself at higher doses to normal (133) and tumor tissue (17) *in vitro*. These facts have led to the search for more potent and less toxic anti-MDR agents for possible clinical use.

## 2b. Verapamil Analogs

An obvious first step in searching for less toxic, more selective chemosensitizing agents is to examine the ability of structural analogs of verapamil to antagonize MDR (Table 1-2).

Kessel and Wilberding (129) studied the anti-MDR effects of the verapamil analog tiapamil, and 13 additional analogs of tiapamil, in a P388/ADR cell clonogenic assay. Tiapamil, which contains a dithiane tetraoxide substituent on the carbon backbone, was 50-fold less potent than verapamil in causing a partial (15-fold) reversal of the 100-fold doxorubicin resistance, while the analog Ro 11-3651 (DMDP) was only 7-fold less potent, and the dithiane substituted analog Ro 11-2933 was 10-fold more potent than verapamil in mediating this effect. The degree of doxorubicin potentiation by the four active tiapamil compounds was found to correlate with their respective effect on drug accumulation (129).

Another group further examined the activity of DMDP in the same cell line, and found that non-toxic doses of this tiapamil analog (3  $\mu\text{M}$ ) and verapamil (6  $\mu\text{M}$ ) caused similar effects on doxorubicin cytotoxicity and accumulation, but that DMDP was 6-fold more toxic to cells than verapamil when used alone ( $\text{IC}_{50} = 14 \mu\text{M}$ ) (175).





A Japanese group reported that a synthetic isoprenoid structurally related to verapamil, SDB-ethylenediamine, caused a 2- to 5-fold increase in doxorubicin cytotoxicity in the P388/ADR cell line, as well as partial reversal of cross-resistance to vincristine, vinblastine and daunomycin (242). Antagonism of MDR was also found in P388/VCR cells and in colchicine selected MDR human epidermal carcinoma KB-Ch<sup>R</sup>-24 (155, 242). Interestingly, this verapamil analog, which has a slightly modified carbon backbone and contains a 9 carbon isoprene side group, has negligible activity as a calcium channel blocking agent compared to verapamil (242).

Thus, certain compounds structurally related to verapamil partially reverse MDR, but lack other physiological effects of verapamil. While of clear mechanistic interest (see Section 4b, below), this also suggests that through structure-activity relationships it may be possible to identify and exploit those structural features necessary for anti-MDR activity, while eliminating those important for antagonism of calcium channels or other interactions.

### *2c. Other Calcium Channel Blockers*

Since a number of calcium channel blockers structurally dissimilar to verapamil have been described and are in clinical use, it occurred to researchers to examine their effect against MDR. While it subsequently became clear that these drugs' pharmacologic effect on slow inward calcium transport was completely unrelated to their anti-MDR activity (see Section 4b, below), many were nevertheless found to be quite active antagonists of MDR (Table 1-2) (96). Indeed, in Tsuruo's early observations of the anti-MDR effects of verapamil, it is also noted that caroverine and prenylamine, agents known to block calcium channels (26, 112), were as active as verapamil in altering the sensitivity of P388/VCR and K562/VCR cells to vincristine and P388/ADM cells to doxorubicin, though 2- to 3-fold less potent (229, 230).

Additional studies specifically examined the effects of calcium channel blockers in MDR cell lines and found that two other classes of structurally dissimilar drugs, diltiazem and the nifedipine analogs, had significant anti-MDR activity (181, 227, 231).



Interestingly, nifedipine itself, known to be a strong calcium channel blocker (63), was a poor antagonist of MDR in P388/VCR and K562/VCR cells (227) and had no effect in potentiating doxorubicin in P388/ADR cells (181). However, the dihydropyridine analogs niludipine, nimodipine, and nicardapine were found to be potent antagonists of MDR, with 3.5 to 10  $\mu$ M nicardapine fully reversing vincristine resistance in P388/VCR and K562/VCR cells (227, 231) and partially reversing doxorubicin resistance in P388/ADR cells (181, 227). These studies pointed out what was later proven to be the distinct pharmacologic effects of these drugs on calcium channels and on MDR (102).

Several groups have shown that perhexiline maleate, another structurally unrelated drug which has among other effects calcium channel blocking activity (62), can also antagonize MDR. Non-toxic concentrations of perhexiline maleate (10  $\mu$ M) caused a slight increase in the sensitivity of a MDR human breast cancer cell line MCF-7Ad to doxorubicin and vinblastine (177), and a more substantial reversal of P388/ADR cell resistance to these same drugs (67), along with concomitant increases in [ $^3$ H]-drug accumulation. Like verapamil, however, perhexiline maleate is a clinically used vasodilator and is an unlikely candidate for the modulation of tumor resistance in humans, due to its dose-limiting hemodynamic side effects.

Finally, in the search for a calcium channel blocker with anti-MDR activity and less vasodilatory activity, bepridil was found to be a potent inhibitor of colchicine selected Chinese hamster ovary cell resistance to doxorubicin (74-fold reversal) (203). Drug concentrations shown necessary for anti-MDR activity *in vitro* (2 to 4  $\mu$ M) by this study had been reported to be clinically achievable without resultant toxicity (20). When studied in the MDR human ovary cell line 2780<sup>AD</sup>, bepridil was equally as active as verapamil, but caused only a 10% increase in sensitivity to doxorubicin. However, of all the calcium channel blockers studied to date, bepridil appears to be the most worthy of continued pre-clinical development for the *in vivo* reversal of drug resistance.



## 2d. *Calmodulin Antagonists*

The second most studied group of anti-MDR agents are drugs which also possess the ability to inhibit CaM-mediated processes, such as the  $\text{Ca}^{++}$ /CaM-dependent form of cyclic nucleotide phosphodiesterase (137). While the activity of CaM antagonists against MDR was first described by Tsuruo (229), Ganapathi has most extensively studied this group of drugs, focusing on the phenothiazine (PTZ) antipsychotic trifluoperazine.

Based on the finding that verapamil functioned as a chemosensitizer in MDR cells, Tsuruo examined several other compounds known to perturb the intracellular calcium environment (Table 1-3). Non-toxic concentrations of the well known CaM antagonist, trifluoperazine, caused a 5- to 10-fold increase in vincristine and doxorubicin sensitivity in 20- and 40-fold resistant P388/VCR and P388/ADM cells, respectively (229), and fully reversed 17-fold K562/VCR resistance to vincristine (230). Like verapamil, trifluoperazine also caused a 4- to 5-fold increase in [ $^3\text{H}$ ]-vincristine and [ $^3\text{H}$ ]-doxorubicin accumulation in these cells, and did not significantly alter drug cytotoxicity or accumulation in their sensitive counterparts (229, 230). Although verapamil appeared to be more effective than trifluoperazine in antagonizing MDR, higher concentrations of verapamil than the CaM antagonist were used (6.6  $\mu\text{M}$  versus 2 to 3  $\mu\text{M}$  trifluoperazine) due to trifluoperazine's greater toxicity when used alone (229). Interestingly, equimolar concentrations of trifluoperazine and verapamil (6.6  $\mu\text{M}$ ) were shown to produce equivalent increases in [ $^3\text{H}$ ]-drug accumulation in all three cell line (229, 230). Unfortunately, no study directly comparing the potency, toxicity and maximal anti-MDR effect of trifluoperazine and verapamil in this or any other cell lines has been reported.

Ganapathi has focused on the effect of trifluoperazine in altering cellular drug resistance, cross-resistance and drug accumulation (Table 1-3). In 100-fold doxorubicin resistant P388/DOX cells (apparently a different MDR subline than Tsuruo's P388/ADM), 4 to 5  $\mu\text{M}$  trifluoperazine caused a 5- to 10-fold increased inhibition of cellular proliferation and cytotoxicity by doxorubicin in MDR, but not sensitive cells (69, 71), as measured by cell growth and clonogenic assays. 5  $\mu\text{M}$  trifluoperazine also partially antagonized the



resistance of a series of L1210 murine leukemia cell lines made variably resistant to doxorubicin (5- to 40-fold), with the magnitude of this effect seemingly related to the degree of acquired resistance (70) (Table 1-3).

Trifluoperazine's effect on cross-resistance is less clear. Similar to experiments with trifluoperazine and doxorubicin, trifluoperazine enhanced the cellular accumulation and cytotoxicity of the anthracycline daunomycin in the 100-fold cross resistant P388/DOX cells, but not in sensitive P388 cells (71). However, trifluoperazine had significant effects on the cytotoxicity of *Vinca* alkaloids in both MDR and sensitive cells, causing 2- to 10-fold increases in the toxicity of vinblastine and vincristine in the 20- and 100-fold cross resistant P388/DOX cells, respectively, as well as in sensitive P388 cells (72). Conversely, trifluoperazine's effect on the accumulation of [<sup>3</sup>H]-vinblastine was not similar in the P388/DOX and P388 sensitive cells, causing a 8-fold versus 2-fold increase, respectively (72). Thus, trifluoperazine's enhancement of *Vinca* alkaloid cytotoxicity does not clearly parallel its effect on cellular drug levels.

In fact, Ganapathi has argued extensively against a correlation between actual intracellular levels of drug following exposure to trifluoperazine and resultant cellular toxicity. For instance, Ganapathi compared the resultant P388/DOX cellular toxicity of similar intracellular doxorubicin or daunomycin concentrations achieved by either treating with 5  $\mu$ M trifluoperazine or by increasing extracellular drug concentration appropriately, and found that cell kill occurred only with trifluoperazine treatment (74). Furthermore, the cellular accumulation of doxorubicin in the absence and presence of 5  $\mu$ M trifluoperazine was examined in a series of L1210 cell lines made 5-, 10-, 20-, and 40-fold resistance to doxorubicin (70). In the absence of trifluoperazine, cellular doxorubicin accumulation progressively decreased with increasing resistance from 15 - 50% of parental L1210 cell accumulation. The presence of 5  $\mu$ M trifluoperazine caused a constant 1.5-fold enhancement of doxorubicin accumulation in each resistant subline, independent of its level of resistance. In contrast, the effect of trifluoperazine in modulating doxorubicin cytotoxicity was dependent on the level of resistance (Table 1-3), with the magnitude of





chemosensitizing activity correlating with the level of resistance. However, if one re-analyzes Ganapathi's data to calculate the percent modulation of total doxorubicin resistance by trifluoperazine in each subline as a function of the total difference in sensitivity from the parental line, rather than purely the fold decrease in resistance, one actually finds a progressive decrease in the percent reversal of resistance by trifluoperazine in increasingly resistant sublines. This finding is in agreement with the calculated decrease in doxorubicin accumulation relative to the total accumulation defect in increasingly resistant sublines.

Obviously, the effects of alterations in drug accumulation are extremely difficult to assess since drug accumulation and retention are only one of many factors influencing the cytotoxicity of chemotherapeutic agents. Compounding this problem is that drug activity tends to be measured in terms of overall cell toxicity, rather than specific effects at a biochemical or molecular level. Even independent of the effects of chemosensitizers, it is not clear that intracellular drug levels are linearly related to cytotoxicity. In fact, this would not be expected, since chemotherapeutic agents often have multiple targets with different affinities through which they mediate their toxicity. For example, the basis for doxorubicin cytotoxicity has been suggested to be due to a combination of cellular effects, including DNA intercalation (53), membrane binding (223), free radical formation (9), transitional metal ion binding (152), and topoisomerase II-mediated DNA strand breaks (221). Such a multitude of targets implies a complex relationship between drug level and effect. In addition, cells themselves have various intrinsic mechanisms for protection from drug toxicity, such as phase I and phase II drug-metabolizing enzymes and various DNA repair enzymes, so that certain threshold levels of drug may be necessary before cell damage can accumulate.

Nevertheless, the disparity between chemosensitizer modulated accumulation and cytotoxicity in Ganapathi's studies suggests that trifluoperazine, a drug known to effect many cellular enzymes and receptors (47, 167, 195) may alter cell sensitivity to drugs in additional ways unrelated to changes in accumulation. Indeed, several investigators have



shown that CaM antagonists such as trifluoperazine modulate cell sensitivity to bleomycin through inhibition of DNA repair mechanisms (33, 134). These results also suggest that certain MDR cells may have multiple mechanisms of resistance, and reversing the accumulation defect alone may not fully reverse cellular drug resistance. In fact, several MDR cell lines have recently been shown to possess alternate mechanisms of resistance in addition to P-glycoprotein, such as increased levels of the glutathione conjugating enzyme glutathione-*S*-transferase in MCF-7/DOX cells and an MDR P388 line (12, 51), and decreased levels of the drug target topoisomerase II in this same P388 line (52).

In summary, certain CaM antagonists, such as trifluoperazine, function as moderately effective chemosensitizers and enhance cytotoxic drug accumulation and retention in MDR cells. Though the PTZs generally are thought to be less active anti-MDR agents than the calcium channel blocker verapamil, a comparison of Tables 1-1 and 1-3 reveal that the concentrations of verapamil used in most studies are generally greater than trifluoperazine, presumably due to the greater toxicity of the latter compound. Since there appears to be a dose-response relationship for chemosensitizers' anti-MDR effect (19, 70, 211), trifluoperazine's significant toxicity may limit its *in vitro* use to a less than maximally effective chemosensitizing dose range. A number of investigators have clearly shown CaM to be a target for the PTZs' cytotoxic actions (87, 113, 239), and it is therefore intriguing to speculate on the possible selectivity for anti-MDR activity over cytotoxicity for this class of agents if CaM is in fact not directly involved in the pharmacologic reversal of MDR. Clearly, more comprehensive studies of the effects and mechanisms for reversal of MDR by CaM antagonists are necessary and indicated.

## 2e. Anthracycline and Vinca Alkaloid Analogs

Soon after the discovery that MDR cells have reduced drug uptake secondary to an increased active efflux mechanism (212), Skovsgaard tested the intriguing and logical possibility that a specific drug efflux pump would be competitively inhibited by an excess of a non-toxic substrate (213). The ideal anthracycline structural analog for this purpose was



*N*-acetyl-daunorubicin, which lacks the free amino-group of daunomycin essential for DNA intercalation, and thus has a lower affinity for DNA resulting in less cytotoxicity and higher cytoplasmic concentrations (247). Skovsgaard found that a 30-fold excess of *N*-acetyl-daunorubicin caused marked inhibition of active daunomycin efflux in MDR, but not sensitive, Ehrlich ascites cells, leading to increased net daunomycin uptake (213). In addition, *N*-acetyl-daunorubicin had a 7-fold lower affinity for both sensitive and resistant cell nuclei in whole cell lysate preparations, and did not compete with [<sup>3</sup>H]-daunomycin binding to DNA. The effect of *N*-acetyl-daunorubicin on cellular resistance to daunomycin was unfortunately not examined *in vitro*, though a 1:20 combination of daunomycin and its structural analog did cause significant increases in the life span of mice with MDR Ehrlich ascites cells inoculated intraperitoneally (i.p.) compared to no effect of either drug alone (213).

Inaba's group subsequently analyzed the chemosensitizing effects of three additional non-toxic anthracycline analogs on MDR cells and found significant enhancement of vincristine toxicity in P388/VCR cells *in vitro* when used in 100-fold excess (111). Interestingly, one of these analogs also partially reversed the cross-resistance of the cells to daunomycin, implying that either a similar drug efflux mechanism affects both anthracyclines and *Vinca* alkaloids, or that the daunomycin analogs competitively inhibited two different mechanisms. Similarly, Inaba also reported that a number of relatively non-toxic *Vinca* alkaloid analogs, such as vindoline, effectively antagonized both the primary resistance and cross-resistance of MDR P388/ADM and P388/VCR cells (110) when used in 1000-fold excess (Table 1-4). These analogs were 5- to 10-fold less potent than the anthracycline analogs as chemosensitizers and as enhancers of cytotoxic drug accumulation. These findings suggest that a single drug-binding site of broad specificity, rather than multiple, independent binding sites, is responsible for cross-resistance between at least the anthracyclines and *Vinca* alkaloids. Indeed, these speculations agree with molecular studies demonstrating that single base mutations within the *mdr1* gene can alter the levels of cross-resistance displayed by the expressed P-gp (40). It would also be of interest to see



whether these non-toxic analogs competitively inhibit the binding to the photoaffinity labelled vinblastine analog  $^{125}\text{I}$ -NASV (2) or the photoactive verapamil analog  $^{125}\text{I}$ -NASAV (197) to P-glycoprotein.

#### *2f. Other Hydrophobic Cationic Compounds*

The search for agents to circumvent MDR has led to the identification of numerous compounds which are not known to be calcium channel blockers or CaM antagonists, and are not otherwise pharmacologically related. Most of these compounds are amphipathic and lipophilic in nature, and share a broad structural similarity that includes a tricyclic ring nucleus separated by some distance from a cationic group. Chemosensitizing agents as diverse as the antiarrhythmics amiodarone (37) and quinidine (226), the alkaloid derivative cepharanthine (209), the lysosomotropic amines chloroquine and propranolol (208, 245, 246), the antimalarial quinacrine (109, 246), the indole alkaloid reserpine (16), and the antiestrogen tamoxifen and structurally related triparanol analogs (67, 178), have been reported to partially overcome resistance and cross-resistance to cytotoxic drugs, and to increase drug accumulation and retention in various MDR cell lines (Table 4). Whether these many types of pharmacologic agents are acting through a common mechanism to antagonize MDR, whether numerous drug-target interactions are capable of mediating this effect, or whether as a result of their degree of hydrophobicity these drugs simply cause non-specific membrane perturbations leading to increased drug accumulation, is presently unclear.

#### *2g. Cyclosporines*

In addition to well-known immunosuppressive and cytotoxic properties, cyclosporines have been described as having chemosensitizing activity in sensitive and MDR tumor cell lines (233). Structurally and pharmacologically quite different than other known chemosensitizers, cyclosporin A (CsA) is an extremely hydrophobic cyclic peptide of 11





amino acids. Its primary immunosuppressive activity is through the specific inhibition of an early stage of T lymphocyte activation, apparently by interaction with the cytosolic receptor protein, cyclophilin (94). Interest in CsA's potential anti-MDR activity was in part due to reports of its CaM binding properties (41), though it was later clearly shown that CsA does not specifically inhibit CaM-mediated processes (85).

Though there were several earlier reports that CsA could potentiate various cytotoxic drugs in tumor cells (132, 163), Slater *et al* first studied its effect in MDR cells (217). It was found that CsA caused a 3- to 4-fold potentiation of daunomycin toxicity in Erhlich ascites cells with a very low level of primary resistance (2-fold) to daunomycin. It is difficult to know whether CsA was antagonizing the MDR process per se, or simply potentiating anthracycline activity in general, since a 2-fold potentiation of daunomycin cytotoxicity was also noted in sensitive Erlich ascites cells (217). Since then, several groups have found CsA to reverse resistance and cross-resistance in MDR, but not sensitive cells (Table 1-5) (89, 232, 234), while others have shown cytotoxic drug potentiation in both sensitive and resistant cells. For example, CsA caused a 10-fold enhancement of doxorubicin toxicity in sensitive Chinese hamster ovary Aux B1 cells, and a 90-fold enhancement in MDR, colchicine selected CH<sup>R</sup>-5 cells (34).

Also unclear is CsA's effect on drug accumulation in MDR cells. Slater *et al* claimed that CsA reversed resistance but had no effect on drug accumulation in a human T cell ALL cell line selected for vincristine resistance, with low level cross-resistance to daunomycin (216). However, daunomycin accumulation rather than vincristine accumulation was measured in this experiment, and since these cells do not display an accumulation defect to daunomycin, it is probable that they were not of the MDR phenotype. Hait *et al* found that while treatment with CsA enhanced the sensitivity to doxorubicin in MDR P388/DOX cells, but not in sensitive P388 cells, it was associated with 40% increased doxorubicin accumulation in both lines (89). Chambers and coworkers reported a similar degree of increased doxorubicin accumulation (50%), along with enhanced doxorubicin cytotoxicity, in both sensitive Chinese hamster ovary cells and MDR CH<sup>R</sup>-5 cells (34). Thus, it appears



that if, in fact, CsA specifically antagonizes MDR, it is not solely due to modification of drug transport. Interestingly, it has recently been reported that CsA itself is accumulated less in the P-gp overexpressing CH<sup>R</sup>-5 cells than the parent Aux B1 line, and that this difference could be reversed with the chemosensitizer verapamil (78). This suggests that CsA may competitively modify MDR in a manner similar to the non-toxic anthracycline analogs, perhaps in addition to other drug potentiating effects.

More carefully designed experiments probing the effects of CsA on both primary and cross-resistance to a wide range of drugs in both sensitive and well-defined MDR cells lines are clearly necessary to determine if the chemosensitizing effects of CsA are unique to the cells of the MDR phenotype, or rather if CsA synergistically enhances the effect of certain drugs in all tumor cell lines. More specifically, it needs to be determined whether CsA is effecting P-gp, cytotoxic drug targets, or both.

The activity of several non-immunosuppressive cyclosporine analogs on MDR is of great interest with regard to their mechanism of action as well as clinical potential. Twentyman first described the anti-MDR activity of three analogs, cyclosporin C, G and H as correlating with their immunosuppressive effects in a human small cell lung cancer line (234). However, this group later found that the non-immunosuppressive cyclosporines W8-032 and 133-243 were actually more effective as chemosensitizers than identical concentrations of CsA (Table 1-5) (232). Similarly, the non-immunosuppressive 11-MeLeu analog has been shown to be less potent, but equally effective as CsA in sensitizing P388/DOX cells to doxorubicin (89). These results suggest that cyclophilin binding is not essential to the cyclosporines' chemosensitizing effects, and that clinical reversal of drug resistance may be possible using analogs which lack the untoward immunosuppressive and nephrotoxic effects of CsA.

In summary, while the cyclosporines clearly modulate drug resistance in MDR cells, it is uncertain whether the mechanism is through reversal of MDR, potentiation of chemotherapeutic drug toxicity, or both. Indeed, CsA has been shown to potentiate a



number of drugs *in vitro* and *in vivo* both in tumor cells and normal tissues (233). If cyclosporine does have additional, specific chemosensitizing activity against MDR cells, it is likely that it is mediated through different mechanisms than most other chemosensitizers, since it does not consistently alter drug accumulation. This leads to the intriguing possibility that CsA, in combination with another chemosensitizing agent, such as verapamil or trifluoperazine, may act synergistically to antagonize MDR.

### 3. *In Vivo Effects of Chemosensitizers*

The goals in studying the *in vitro* effects of chemosensitizers on MDR are twofold: to further elucidate the mechanism(s) involved in MDR through its antagonism, and to identify and characterize compounds which may act as effective, non-toxic modulators of intrinsic or acquired chemotherapeutic drug resistance in clinical tumors. A number of agents identified as having anti-MDR activity *in vitro* have been tested in mouse tumor models with varying success, and intense interest surrounds the results from current and proposed clinical trials of these agents in humans.

The potential utility of any chemosensitizing agent *in vivo* depends both on its ability to potentiate the cytotoxicity of anti-cancer drugs at clinically achievable concentrations, and its associated toxicity and unwanted side effects. In addition, to obtain the desired therapeutic gain by treatment with chemosensitizers, it is crucial that the enhanced toxicity against cancer cells is not accompanied by a similar enhancement of drug toxicity to host tissue cells. Since an ideal chemosensitizer which specifically and selectively modifies tumor cell sensitivity to cytotoxic drugs without intrinsic toxicity or other physiologic effects has not been identified, it is necessary to carefully test the effect of the potential chemosensitizers in appropriate animal models before human trials begin.

#### 3a. *Effects of Chemosensitizers in Murine Tumor Models*

The role of murine tumor models in identifying efficacious anti-cancer drugs and regimens is well documented (145). The standard *in vivo* model for the study of



pharmacologic reversal of MDR has been measurement of the effect on life span of combinations of cytotoxic and chemosensitizing drugs, injected i.p. daily for 10 days into mice inoculated i.p. with  $1 \times 10^5$  to  $1 \times 10^6$  sensitive or MDR ascites tumor cells (214). A number of the chemosensitizing agents discussed in the preceding sections have been shown to cause an increase in the mean or median life span (ILS) in this model (Table 1-6). For instance, daily i.p. administration of verapamil at doses of 50 to 100 mg/kg caused approximately 30 to 50% ILS in P388/VCR-bearing mice treated with vincristine or vinblastine (227, 228, 242), 25 to 40% ILS in P388/ADR-bearing mice treated with doxorubicin (175, 227), and 120% ILS in daunorubicin resistant Ehrlich ascites-bearing mice treated with daunorubicin (215). Doses of verapamil greater than 125 mg/kg proved toxic when given to mice, particularly in combination with doxorubicin or vincristine (228).

Other calcium channel blockers have shown similar activity *in vivo* (Table 1-6). Accordingly, daily treatment with 125 mg/kg diltiazem, 75 mg/kg nicardipine, or 75 mg/kg niludipine in combination with doxorubicin or vincristine caused 30 to 50% ILS in P388/DOX and P388/VCR-bearing mice (224, 227).

Two of the structural analogs of verapamil which possessed chemosensitizing activity *in vitro* have also been tested *in vivo*. The synthetic isoprenoid SDB-ethylenediamine caused up to a 48% ILS in P388/VCR-bearing mice in combination with vincristine (242). However, 15 to 60 mg/kg of the tiapamil analog DMDP was not effective in potentiating doxorubicin activity in P388/ADR-tumor bearing mice (175). In fact, DMDP caused a decrease in the mean survival time, presumable due to enhanced toxicity when in combination with doxorubicin, since measurement of intracellular drug levels in ascites tumor cells revealed adequate anti-MDR concentrations of the chemosensitizer.

The chemosensitizers quinidine and quinacrine also possess noteworthy activity *in vivo* (109, 226), with the latter causing a 85% ILS in the P388/VCR model. The anthracycline analog, *N*-acetyl-daunorubicin, which possessed no demonstrable toxicity when given in a ratio of 20:1 with daunorubicin, caused a 53% ILS in mice bearing Ehrlich ascites tumors resistant to daunorubicin (213).





There has only been one report of the activity of CaM antagonists in reversing MDR in mouse tumor models. Tsuruo *et al* (227) found that the relatively low dose of 20 to 30 mg/kg clomipramine, in combination with vincristine or doxorubicin, caused a 31 to 33% increase in mean survival time of mice bearing P388/VCR or P388/ADM tumors. There have been no published results of the effect of PTZ CaM antagonists on MDR tumors in mice, in contrast to the extensive work done *in vitro* with these compounds, suggesting that this class of chemosensitizers has proven too toxic to be used effectively in animal studies.

By far the most successful *in vivo* murine trial of a chemosensitizer, to date, has been with cyclosporin A. Slater *et al* found that up to 80 mg/kg of CsA could be given to mice without toxicity, and that in combination with 0.3 mg/kg daunomycin daily for 5 days, caused an ILS of more than 200% in daunorubicin resistant Ehrlich ascites tumor bearing mice, with 10 out of 10 mice treated surviving for greater than 60 days and apparently cured (217). 80 mg/kg CsA or 0.3 mg/kg daunomycin, when used alone, caused only a 26% ILS and 11% ILS, respectively, in the MDR murine model, while daunorubicin alone also cured mice bearing sensitive Ehrlich ascites cells. Interestingly, when the dose of CsA was sequentially reduced, as little as 5 mg/kg CsA together with daunomycin still produced an impressive 169% ILS in the MDR tumor bearing mice.

While these *in vivo* results provide an important corollary to the *in vitro* study of chemosensitizers, the relative simplicity of the experimental design employed ignores many critical factors which contribute to clinical effectiveness. For example, since many chemosensitizers are metabolized by the liver, i.p. injection of these drugs leads to extensive first-pass metabolism. Also, the pharmacokinetics of chemosensitizers has been poorly studied. Measurements of the intracellular drug concentrations achieved in tumors, in addition to steady state plasma concentrations, are important for optimizing treatment regimens, since most lipophilic chemosensitizers are rapidly sequestered within tissue and plasma cells. The pharmacokinetics and anti-MDR activity of chemosensitizer metabolites also deserve attention, since they may or may not possess significant activity themselves.



The use of human tumor xenografts in nude mice has proven to be a valuable model for the study of chemotherapeutic drugs in human disease (77). This system may also prove advantageous for study of the effects of chemosensitizers *in vivo*, through use of either previously selected, well-characterized MDR human tumors, or sensitive human tumors which have been previously studied and propagated in mouse models, into which a cloned human *mdr1* cDNA is transfected. The advantage of the latter is that drug sensitivity and optimal treatment of the parent line would already be known, and drug resistance in the MDR line would be entirely due to expression of the human *mdr1* gene. Inoculation of tumors in extraperitoneal, subcutaneous sites also enables a more careful measurement of tumor response to anti-MDR treatment, and investigation of intracellular drug accumulation.

In summary, experiments performed using simple mouse tumor models have thus far suggested that the clinical reversal of drug resistance may be feasible. However, it is critical to the eventual development of successful chemosensitizer protocols in humans that more carefully designed, sophisticated trials of these agents be done in mice, analyzing all the available data to optimize the treatment regimens and better screen for activity.

### *3b. Effect of Chemosensitizers in Human Trials*

The clinical experience with chemosensitizers for the modification of acquired or intrinsic drug resistance in human tumors is as yet extremely limited. The few trials reported to date have been problematic due to the significant toxicity of chemosensitizer treatment together with the lack of adequate diagnostic analyses verifying tumor resistance mechanisms. For example, attempts to combine verapamil with single agent chemotherapy have so far failed, due to the intolerable cardiac effects sustained at levels of verapamil nearing the *in vitro* anti-MDR range (3 to 6  $\mu\text{M}$ ). In a phase I study of escalating doses of verapamil plus 1.5  $\text{mg}/\text{m}^2$  vinblastine for 5 days in 17 patients with advanced malignancies unresponsive to unspecified chemotherapy, no objective tumor responses were noted, and the majority of patients developed significant ECG changes, including first-degree heart block and junctional rhythms (21). Peak plasma verapamil concentrations achieved during



the constant i.v. infusion (0.12 mg/kg/hr) were 0.45  $\mu$ M, well below the concentrations necessary to produce anti-MDR effects *in vitro* (Table 1-1). Similarly, a pilot study of oral verapamil and Adriamycin (50 mg/m<sup>2</sup> every 3 weeks) in 13 patients with chemotherapy resistant tumors (8 to Adriamycin) was limited by verapamil induced nausea, hypotension, and cardiac arrhythmias (173). While one partial response and two minor responses were noted in patients who had received prior Adriamycin, plasma or tissue levels of Adriamycin or verapamil were not measured. However, it is unlikely that oral verapamil administration resulted in the necessary anti-MDR levels, based on other reports of the pharmacokinetics of verapamil (68). In neither of these studies did verapamil appear to potentiate the cardiac toxicity or myelosuppression of chemotherapeutic drug treatment.

Finally, a trial of escalating dose i.v. verapamil plus Adriamycin (50 mg/m<sup>2</sup>) in 8 patients with refractory ovarian cancer again produced unacceptable toxicity, manifested by severe hypotension, heart block and congestive heart failure, without objective response to therapy (165). However, since none of the patients had received previous Adriamycin or other natural product drugs, but rather combinations of cyclophosphamide and cisplatin, there was no clear reason to believe that their tumor resistance involved the MDR mechanism. The intensive verapamil infusion (9  $\mu$ g/kg/min) led to median and peak plasma verapamil levels of 2 and 5  $\mu$ M, respectively. Though these concentrations are within the range of experimentally relevant anti-MDR concentrations, verapamil is clearly too toxic in humans to be used in this manner and at these doses.

In a recently reported trial of the CaM antagonist, trifluoperazine, plus doxorubicin, 36 patients with either acquired or intrinsic tumor resistance to prior doxorubicin therapy were evaluated (149). 60 mg/m<sup>2</sup> doxorubicin was given as a constant infusion, with an escalating oral dose of trifluoperazine from 20 mg/day to 100 mg/day. Interestingly, of the one complete response and 6 partial responses that were seen, all were in patients who had acquired, rather than intrinsic, tumor resistance to doxorubicin. Dose-limiting side effects of this regimen were the extrapyramidal effects associated with trifluoperazine, such as motor restlessness, akathisia, facial dystonia, and resting tremor. Trifluoperazine plasma



levels ranged widely, but were all less than 0.3  $\mu\text{M}$ , and thus at least 10-fold less than those found optimal *in vitro* (Table 1-3).

The major problem with all of these studies is that the concentrations of chemosensitizers at which maximum activity is found *in vitro* are clinically unachievable without severe toxicity and side effects. An additional flaw, however, is that the mechanism of tumor resistance in patients has not been determined. With the recent advent of molecular diagnostic techniques, measurement of P-gp expression and *mdr1* mRNA levels using immunologic and genetic probes will better define the potential for anti-MDR chemosensitizer treatment in specific patients. As more specific and potent chemosensitizers with less clinical toxicity and side effects enter clinical trials, it will be critical to carefully plan and coordinate therapeutic and diagnostic procedures, by measuring MDR markers before and after treatment, and analyzing both tumor and plasma drug pharmacokinetics.

#### 4. Mechanism of Action of Chemosensitizers in Multidrug Resistance

The second major goal of studying chemosensitizers is to better understand the molecular and biochemical mechanism of MDR through its pharmacologic reversal. Given the extremely broad range of compounds which have been shown to effectively alter MDR, it is difficult to envision a single common cellular mechanism or target through which these actions are mediated. It is possible that a number of interactions with different cellular targets may result in a similar effect on cytotoxic drug potentiation and accumulation. However, a specific mechanism of action has not been clearly elucidated for a single chemosensitizer. The following sections will review a number of hypothetical mechanisms which have been suggested to be relevant to chemosensitizers' anti-MDR action.

##### 4a. Effects on Membrane Properties

Several investigators have made observations regarding differences in plasma membrane order or structure in MDR versus sensitive tumor cells. For instance, Ramu *et*





*al* have noted that P388/ADR cells have altered cellular lipid content and composition, and possess a higher degree of plasma membrane lipid structural order than their sensitive counterparts (179, 180). Similarly, Seigfreid found that anthracycline resistance in sarcoma 180 cells correlated with progressive increases in membrane fluidity (210). It has been suggested that these membrane differences may contribute to the alterations in drug accumulation seen in MDR. Since cationic, amphipathic drugs, like most chemosensitizers, are known to interact with polar lipids such as phosphatidylserine (141), it is speculated that chemosensitizers may cause perturbations in MDR membrane order or fluidity, resulting in further changes in cellular drug accumulation properties.

As a model of the possible effect of membrane perturbation or destabilization on MDR, several investigators have shown that the surface active, nonionic detergent Tween 80 caused alterations in drug accumulation and cytotoxicity quite similar to verapamil, and that this effect was seen in MDR, but not in sensitive P388 cells (131, 185). However, using a different MDR P388 cell line, Inaba and colleagues could not demonstrate this effect by Tween 80 (106, 107).

In further studying the possible membrane related effects of chemosensitizers, Ramu found that perhexiline maleate, a compound known to raise phospholipid content in fibroblasts (6), also functioned to reverse MDR (177). While this was cited as evidence favoring a membrane based mechanism of action, perhexiline maleate is also known to block calcium channels (62), resulting in vasodilatory activity similar to verapamil and other calcium channel blockers known to reverse MDR.

The "non-specific" membrane effects of PTZ CaM antagonists have also received a great deal of attention, both with respect to their anti-MDR activity and their antipsychotic and cytotoxic actions (191, 204). Indeed, a number of other unrelated chemosensitizers are similar to the PTZs in that their high degree of lipophilicity results in a preferential partitioning into membrane structures (123). However, this property certainly does not preclude their significant interaction with membrane proteins such as P-gp, or other intracellular targets, in a specific, if yet undefined, manner.



Thus, it is not known at this time how the structural organization of MDR membranes or their alteration by chemosensitizers may relate, if at all, to changes in drug accumulation properties. It may be that certain chemosensitizers act within the membrane to somehow alter the local environment in the vicinity of P-glycoprotein, leading to structural modification and inhibition of drug efflux. Alternatively, overexpression of the membrane protein P-gp may directly cause the alterations in membrane properties noted in MDR cells, and these changes may simply be an epiphenomenon unrelated mechanistically to drug accumulation.

#### *4b. Alteration in Calcium Homeostasis*

The initial discovery that verapamil and other calcium channel blockers could antagonize MDR (227, 228, 229) prompted the suggestion that calcium may play a role in the development or maintenance of MDR, perhaps through a calcium-dependent efflux mechanism, and that chemosensitizers may function by altering calcium fluxes. However, mounting evidence indicates that alterations in calcium homeostasis do not effect MDR. For example, several investigators have shown that P388 sensitive and MDR cells have similar levels of total intracellular  $\text{Ca}^{++}$  content (128, 153). Furthermore, a number of groups have demonstrated conclusively that unlike excitable tissues, neither MDR cells nor their sensitive counterparts possess calcium channels, since calcium uptake was not altered by membrane voltage potentials or calcium channel blockers in MDR or sensitive cells P388, Chinese hamster ovary or C<sub>6</sub> glioblastoma cells (29, 102, 128, 181). Similarly, whole-cell and single-channel patch-clamp techniques demonstrated that both drug sensitive CCRF-CEM and MDR CEM/VLB<sub>100</sub> cells lack voltage-gated calcium channels (135).

Even more compelling evidence against calcium fluxes effecting MDR was provided by two studies which pharmacologically manipulated calcium levels to mimic the effect of calcium channel blockers in those excitable tissues which express calcium channels. Accordingly, treatment of MDR C<sub>6</sub> cells with manganese ion, which produces a reduction



in calcium uptake by a non-competitive inhibition of calcium fluxes, or the calcium ionophore A23187, which causes increased calcium uptake through artificial channels, had no influence on doxorubicin accumulation or cytotoxicity (102). Similar results were found by alteration in net intracellular calcium by reduction of extracellular calcium concentrations or chelation of calcium ions by EGTA (177). In all of these states of altered calcium homeostasis, verapamil maintained its anti-MDR activity without change. Thus, it is apparent that alterations in calcium flux do not alter the level of drug resistance in MDR or sensitive cells, and that calcium channels are not the target of chemosensitizers in this system.

#### *4c. Inhibition of Calmodulin Mediated Processes*

Similar to the situation for calcium channels blockers, the finding that PTZs and other CaM antagonists modulate MDR implicated CaM in the regulation or reversal of MDR. However, definitive evidence as to the role of CaM-mediated events in MDR is lacking. It is known that CaM is involved in the regulation of a wide variety of critical cellular functions (144). Particularly relevant to MDR is CaM's role as an activator of plasma membrane  $\text{Ca}^{++}$ -ATPase ion pumps (81, 159), and as a stimulator of protein kinase mediated phosphorylation and dephosphorylation (201, 202), all processes which are inhibited by CaM antagonists (176, 241). Whether CaM may activate or regulate the function of P-gp in MDR is a provocative, but unanswered, question.

Attempts to compare CaM levels in sensitive versus MDR cells found no gross differences levels between P388 and P388/DOX cells (153), nor between CEM and CEM/VLB<sub>100</sub> cells (13). Whether the primary structure or function of CaM in MDR cells is in any way altered is not known. Several investigators have attempted to correlate the potency of CaM antagonists as inhibitors of CaM-mediated processes with their chemosensitizing activity in MDR cells, with conflicting results. For example, Ganapathi studied three PTZ CaM antagonists, trifluoperazine, prochlorperazine and chlorpromazine, and the naphthalene-sulfonamides W12 and W13 in P388/DOX cells, and found that their



potency in this system was in general agreement with their relative anti-CaM potency (73). Accordingly, trifluoperazine was more potent and more effective at equimolar concentrations than prochlorperazine and chlorpromazine, and W13 was more effective than the poor CaM inhibitor W12. Unfortunately, a statistical analysis of the degree or significance of correlation between anti-CaM and anti-MDR activity was not performed, nor were structural analogs or metabolites lacking CaM inhibitory properties examined. It is of interest that W12 and W13, drugs with similar hydrophobicity and structure (99), displayed relatively different effects on MDR (Table 1-3), implying that non-specific hydrophobicity alone is not sufficient to alter MDR, as some have suggested for inhibition of CaM (192). However, both Tsuruo (229) and Akiyama (5) reported that the chemosensitizing activity of trifluoperazine, thioridazine and chlorpromazine in KB-Ch<sup>R</sup>-24 cells, respectively, did not correlate with known IC<sub>50</sub>'s for the inhibition of CaM by these drugs. Furthermore, Akiyama found that the potent CaM antagonists W7 and W5 were equally poor chemosensitizers, although W7 was a significantly more potent CaM antagonist (99). Clearly, the sample size in each of these studies was far too small to make any definite conclusions regarding the role of CaM antagonism in the modulation of MDR. In addition, the well known "non-specific" nature of PTZs and other CaM antagonists make attempts to analyze their effects on whole cells extremely difficult for any system. Nevertheless, a more rigorous and systematic study of the pharmacologic relationship between CaM antagonists and reversal of MDR is certainly warranted. While a positive correlation would not directly implicate CaM's role in MDR, it may suggest that anti-CaM activity is a useful marker for chemosensitizer activity, possibly leading to the identification of novel modifiers of MDR.

#### *4d. Inhibition of Protein Kinase C*

A great deal of provocative evidence has been reported implicating a possible regulatory role for the phospholipid/Ca<sup>++</sup>-dependent protein kinase (PKC) in MDR, suggesting that interaction of chemosensitizers with PKC may be important. For example, a number of chemosensitizers, such as the PTZs and verapamil, are also known to inhibit the activity of





PKC in isolated systems (150, 200). Several groups have found increased levels of PKC in the MDR human breast cancer line, MCF-7/DOX (59, 166), and preliminary evidence suggests that the pattern of expression of PKC isoforms may be altered in MDR cells (1, 8). Treatment of a variety of cell lines with activators of PKC, such as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), results in transient increases in cellular drug resistance as well as changes in drug accumulation. Specifically, treatment of sensitive human KB cells with TPA caused a transient, 2-fold protection against the cytotoxicity of VP-16, vincristine and mitoxantrone, which could be circumvented by co-administration of verapamil (58). Similarly, phorbol ester increased the resistance of both sensitive MCF-7 and MDR MCF-7/DOX cells to doxorubicin and vincristine (59), and increased the resistance of P388, though not P388/DOX, cells to daunomycin (127). Furthermore, the relatively specific PKC inhibitor, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), inhibited the phorbol ester induced decrease in P388 cell vincristine uptake (105).

Since the activation of PKC by phorbol ester in MDR and other cell types results in the phosphorylation of several proteins possibly associated with MDR (7, 59), it is intriguing to speculate on the possible modulation of P-gp function by PKC. Indeed, it has been reported that phorbol ester causes an increase in P-gp phosphorylation (90). Paradoxically, verapamil and trifluoperazine also have been reported to cause a hyperphosphorylation of P-gp, though apparently at different residues (32). Whether the effect of chemosensitizers on P-gp phosphorylation states is mediated by PKC is presently not known.

Thus, it appears that within the calcium messenger system, PKC is a target for many chemosensitizers, and may mediate the regulation or modulation of MDR. However, a great deal of work is necessary to better define the doubtlessly complex regulatory mechanisms effecting P-gp before the role of chemosensitizers in altering or modulating the system may be determined.



#### 4e. Interaction with P-glycoprotein

In the preceding sections, several theories have been discussed regarding the possible modulation of MDR by the known interactions of certain chemosensitizers with aspects of the calcium mediated second messenger system. It is also quite possible that chemosensitizers act by directly blocking or altering the function of P-gp, and in fact, several lines of evidence suggest that this may be the case for certain anti-MDR agents. First, with the recent discovery of P-gp and its putative role as a membrane drug efflux pump (38), it is important to recall the earlier studies demonstrating the chemosensitizing effect of non-toxic anthracycline and *Vinca* alkaloid analogs (110, 213) (discussed in Section A.2e). The apparent competitive inhibition of drug efflux in MDR cells displayed by these compounds suggests that one or more specific drug binding sites may exist in P-gp, and that agents which bind to these sites with similar or greater affinity inhibit P-gp mediated cytotoxic drug efflux and result in increased cellular cytotoxic effect.

More recent evidence that cytotoxic drugs and some MDR antagonists bind to P-gp came from experiments with preparations of P-gp enriched MDR membrane vesicles. Cornwell *et al* first noted that [<sup>3</sup>H]-vinblastine bound with an affinity constant of 1.5  $\mu$ M in a specific, saturable, temperature-dependent manner to membrane vesicles from MDR KB-C4 cells, but did not bind to sensitive KB-3-1 or revertant KB-R1 cell membranes (43). This binding was strongly inhibited by excess unlabeled vinblastine, vincristine, and verapamil, less so by daunomycin, and poorly inhibited by colchicine (43). Safa and others used photoactive analogs of vinblastine ([<sup>3</sup>H]-NABV and <sup>125</sup>I-NASV) which irreversibly bind to MDR cell membrane fractions (45), to identify vinblastine acceptor proteins (198). A 150 - 180 kDa photoaffinity labelled membrane protein was found specifically in MDR cell lines and was immunoprecipitated with a polyclonal antibody known to cross-react with P-gp from other cells. In addition, verapamil inhibited <sup>125</sup>I-NASV photoaffinity labelling of a 170 kDa KB-V1 cell membrane protein which was immunoprecipitated with a monoclonal antibody to P-gp (44). Further studies of the competitive binding of chemosensitizers to P-gp found that (i) KB-V1 MDR vesicles bound 15- to 20-fold more verapamil and diltiazem



than sensitive KB-3-1 vesicles, in a specific and saturable manner (44), (ii) the chemosensitizers verapamil, nicardapine, quinidine, and to a lesser extent trifluoperazine, inhibited [ $^3\text{H}$ ]-vincristine binding to K562/ADM membrane vesicles (154), and (iii)  $^{125}\text{I}$ -NASV photoaffinity labeling of KB-V1 and CEM/VLB<sub>1K</sub> P-gp was effectively inhibited by 10 to 12.5  $\mu\text{M}$  concentrations of reserpine, cepharanthine, quinidine, and the isoprenoid SDB-ethylenediamine, but poorly inhibited by up to 100  $\mu\text{M}$  concentrations of the PTZ chemosensitizers trifluoperazine, thioridazine and chlorpromazine (2, 16). These results strongly suggest that certain chemosensitizers, such as the calcium channel blockers and structurally related isoprenoids, compete with the *Vinca* alkaloids for a common P-gp binding site. Additional experiments with photoactive, radiolabelled verapamil analogs, such as [ $^3\text{H}$ ]-azidopine and  $^{125}\text{I}$ -NASAV, further implicate a common acceptor for these drugs, since nifedipine, nicardapine, verapamil and vinblastine effectively inhibit their irreversible binding to P-gp, but trifluoperazine, chlorpromazine, doxorubicin and colchicine do not (196, 197, 199, 243).

In addition, MDR cell membrane vesicles have been shown to inwardly transport [ $^3\text{H}$ ]-vinblastine in an ATP-dependent fashion (101). Using this simplified model of MDR, investigators have shown that vinblastine transport is inhibited by competitive and non-competitive inhibition of ATP, as well as by vinblastine, vincristine, verapamil, quinadine, and less potently by daunomycin and colchicine.

A fourth, independent line of evidence that chemosensitizers may interact with P-gp came from the recent purification and partial characterization of P-gp by Hamada and Tsuruo (92, 93). Enzymatically active P-gp was purified from human K562/ADM cells by immunoaffinity chromatography, and found to possess ATPase activity (93). Interestingly, while vincristine and doxorubicin did not affect the ATPase activity of immobilized P-gp, the chemosensitizers trifluoperazine and verapamil caused increased activity (92). Since PTZs are known to inhibit the activity of other ATPases (167, 176), it is possible that trifluoperazine interferes with a P-gp mediated drug efflux mechanism in a similar manner, although it is paradoxical as to why an increase is seen in ATPase activity. It is anticipated



that further studies of the ATPase function of P-gp will focus on its possible role as a target for chemosensitizers.

Thus, it appears that there are at least several sites on P-gp through which chemosensitizers may interfere with the MDR process. Since a number of chemotherapeutic drugs encompassed by the MDR phenotype, such as doxorubicin, daunomycin and colchicine, do not appear to competitively bind to the calcium channel blocker/vinblastine acceptor on P-gp, it is possible that there may be other independent drug binding sites within the same molecule that lead to active efflux, or that a family of drug binding proteins may exist, which display different affinities for various classes of compounds. Chemosensitizers such as the PTZs may specifically bind to these additional sites and antagonize MDR, although this cannot explain why such agents most often similarly affect *Vinca* alkaloid, anthracycline and colchicine drug accumulation and resistance. Alternatively, PTZs and other chemosensitizers may inhibit P-gp activity by interacting with physically separate sites on P-gp, such as the ATPase or phosphorylation domains, or act by altering the local membrane environment leading to structural or functional changes in P-gp.

#### *4f. Inhibition of Lysosomal Function*

The demonstration by Akiyama and coworkers that verapamil affected the function of lysosomes by inhibiting their degradation of cellular proteins (3, 4), led to the discovery that certain other lysosomotropic agents, such as propranolol (226) and chloroquine (208, 245), could also partially reverse MDR. These findings, together with the fact that cytotoxic concentrations of vinblastine or doxorubicin caused an increased number of lysosomal-like vacuoles in cells (245), suggested an alternative hypothesis for the cellular mechanism of MDR, proposed by both Beck (14) and Sehested *et al* (205). This theory can be summarized as follows: lysosomes or other intracellular acidic compartments in MDR cells protonate and entrap weakly basic drugs such as doxorubicin or vinblastine, which are then





extruded from the cells by vesicular fusion with the plasma membrane. In this scheme, P-gp serves not as a drug efflux pump, but rather to alter or direct membrane turnover or vesicular trafficking (14). Since verapamil and chloroquine appear to disrupt lysosomal functions, they may therefore interrupt the ability of vesicles to transport drug out of cells, resulting in increased drug reaching its cytotoxic target. In support of this, the microfilament-disrupting agent, cytochalasin B, also inhibited drug efflux and enhanced cytotoxicity, though in sensitive as well as MDR cells (130, 225). Recently, however, Beck's group reported that a number of additional lysosomotropic amines, such as methylamin, suramin, trypan blue, and epinephrine, did not potentiate the cytotoxicity of vincristine in MDR CEM/VLB<sub>100</sub> cells (246), from which it was concluded that the ability to impair lysosomal function was not sufficient to alter MDR. It may be that lysosomes function as an alternative, unrelated but reversible mechanism for drug detoxification in both sensitive and MDR cells, although this remains to be determined.

## 5. *Future Directions*

The preceding sections demonstrate that while a tremendous amount of data exists regarding the effects of a host of chemosensitizers on the cellular pharmacology of MDR cell lines, very little is yet known about their mechanism of action. Since such a wide variety of agents possessing remarkably varied cellular effects reverse MDR, it is clearly necessary to carefully study the anti-MDR activity of drugs within a single pharmacologic class, attempting to define their possible cellular targets and the structural features which enhance their interaction with this target. Indeed, if specific structural features determine anti-MDR activity, rather than non-specific properties, it is likely that their mechanism of action is mediated by classical receptor-ligand interactions, implying that the rational design of structurally optimal agents may result in increased specificity and potency.

To this end, systematic and statistically significant structure-activity relationships of the chemosensitizing effects of individual classes of pharmacologic compounds need to be performed to:



- (i) Define the specific structural features, if any, necessary for the pharmacological reversal of MDR, and possibly identify an optimal 'pharmacophore' for this activity or target
- (ii) Identify the relevant target(s) for the chemosensitizing effects of a particular class of drugs and characterize the drug-target interactions
- (iii) Identify more specific, potent and less clinically toxic drugs with which to possibly antagonize MDR in a clinical setting
- (iv) Better define and elucidate the molecular mechanism of MDR itself
- (v) Identify the natural substrate for P-gp and define its biological role.

The present study addresses the first three of these points. Specifically, systematic structure-activity relationships for the chemosensitizing effects of a series of closely related PTZ CaM antagonists and structurally related compounds were performed, and the role of non-specific hydrophobic effects versus specific interactions with CaM assessed. Based on the structural determinants found necessary for PTZ anti-MDR activity, a novel class of chemosensitizing agents was identified and their effect on MDR *in vitro* and *in vivo* was extensively characterized. In addition, initial steps were taken toward characterizing their mechanism of action for the antagonism of MDR.



TABLE 1-1

Summary of Studies Using Verapamil to Reverse Multidrug Resistance

[Verapamil] ( $\mu$ M)	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
6.6	P388/VCR	VCR (31)	122	Tsuruo, 1981
		VLB (7)	7	
2.2		VCR (31)	34	
		VLB (7)	7	
3.3	P388/VCR	VCR (20)	26	Tsuruo, 1982
6.6			84	
10	P388/VCR	VCR (20)	40	Tsuruo, 1985
		VLB (12)	7	
3.3	P388/ADM	DOX (43)	12	Tsuruo, 1982
6.6			13	
10	P388/ADR	DOX (27)	9	Tsuruo, 1985
		DAU (19)	9	
30	P388/ADR	DOX (40)	28	Ramu, 1984
10	P388/ADR	DOX (51)	11	Klohs, 1986
		DAU (38)	9	
10	P388/DOX	DOX (37)	8	Kramer, 1988
6	P388/ADR	DOX (100)	11	Radel, 1988
40	EA/DAU	DAU (5)	4	Slater, 1982
6.6	K562/VCR	VCR (17)	61	Tsuruo, 1982
		DOX (3)	4	
6	C6/DOX	DOX (20)	10	Huet, 1988]
1	CHO/B30	DAU (100)	8	Cano-Gauci, 1987
10	8226/DOX40	DOX (50)	40	Bellamy, 1988
6	MES-SA/Dx5	DOX (100)	7	Harker, 1986
		DAU (100)	14	
		MITO (60)	8	
		ActD (1200)	9	
		VLB (100)	1	
		VCR (240)	1	

(Continued)



**TABLE 1-1**  
(Continued)

[Verapamil] ( $\mu$ M)	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
10	CEM/VLB <sub>100</sub>	VLB (420)	22	Zamora, 1988
10	CEM/VLB <sub>1K</sub>	VLB (930)	21	Beck, 1988
10	MCF-7/DOX	DOX (100) VCR (100)	10 10	Fine, 1988
20	MCF-7/DOX	DOX (100)	7	Kramer, 1988
20	KB-Ch <sup>R</sup> 8-5-11-24	CH (220) DOX (21) VLB (23) VCR (73)	63 23 38 183	Fojo, 1985
2	2780 <sup>AD</sup>	DOX (100)	4	Schuurhuis, 1987
1	2780 <sup>AD</sup>	DOX (170)	6	Rogan, 1984
1	1847 <sup>AD</sup>	DOX (5)	6	
22	DC-3F/AD	DOX (100) ActD (4200)	20 85	Delaporte, 1988
6.6	NCI-H69/LX4	DOX (85) VCR (750)	19 72	Twentyman, 1986
	MOR/DOX	DOX (12)	5	
	COR-L23/DOX	DOX (12)	4	
10	HCT-8	DOX (NE)	4	Klohs, 1988
	Colon 26	DOX (NE)	4	
	LoVo	DOX (NE)	3	

*Abbreviations:* ActD, actinomycin D; CH, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; EA, Ehrlich ascites; MITO, mitoxantrone; VCR, vincristine; VLB, vinblastine.





TABLE 1-2

**Summary of Studies Using Verapamil Analogs and Other Calcium Channel Blockers to Reverse Multidrug Resistance**

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Tiapamil	500	P388/ADR	DOX (100)	15	Kessel, 1985
DMDP	20	P388/ADR	DOX (100)	15	Kessel, 1985
	3	P388/DOX	DOX (100)	19	Radel, 1988
Tiapamil Analog (Ro-2933)	1	P388/ADR	DOX (100)	15	Kessel, 1985
SDB-ethylene- diamine	34 $\mu$ g/ml	KB-Ch <sup>R</sup> -24	DOX (28) VCR (89)	35 55	Nakagawa, 1986
	100 $\mu$ g/ml	P388/ADM	DOX (37)	3	Yamaguchi, 1986
			DAU (19)	2	
			VCR (17)	3	
			ActD (10)	4	
			VCR (8) DOX (10)	33 10	
Diltiazem	20	P388/ADR	DOX (51)	3	Klohs, 1986
	35	K562/VCR	VCR (21)	9	Tsuruo, 1983a
		P388/VCR	VCR (20)	29	Tsuruo, 1985
			VLB (12)	7	
		P388/ADR	DOX (27)	9	
			DAU (19)	5	
	100	P388/VCR	VCR (15)	29	Tsuruo, 1983b
		P388/ADM	DOX (49)	16	
Nifedipine	3	P388/ADR	DOX (50)	1	Ramu, 1984c
	100	P388/VCR	VCR (15)	12	Tsuruo, 1983b
		P388/ADM	DOX (49)	4	
Niludapine	10	K562/VCR	VCR (21)	48	Tsuruo, 1983a
		P388/VCR	VCR (15)	26	Tsuruo, 1983b
		P388/VCR	VCR (20)	25	Tsuruo, 1985
			VLB (12)	7	
		P388/ADM	DOX (49)	10	Tsuruo, 1983b
		P388/ADM	DOX (27)	10	Tsuruo, 1985
			DAU (12)	3	

(Continued)



**TABLE 1-2**  
(Continued)

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Nimodipine	35	K562/VCR	VCR (21)	54	Tsuruo, 1983a
		P388/VCR	VCR (15)	26	Tsuruo, 1983b
		P388/ADM	DOX (49)	15	
Nicardapine	3	K562/VCR	VCR (21)	25	Tsuruo, 1983a
		P388/VCR	VCR (15)	33	Tsuruo, 1983b
		P388/ADM	DOX (49)	4	
		P388/ADR	DOX (50)	4	Ramu, 1984c
	10	K562/VCR	VCR (21)	52	Tsuruo, 1983a
		P388/VCR	VCR (15)	70	Tsuruo, 1983b
		P388/VCR	VCR (20)	70	Tsuruo, 1985
			VLB (12)	7	
		P388/ADM	DOX (49)	27	Tsuruo, 1983b
		P388/ADR	DOX (27)	27	Tsuruo, 1985
			DAU (19)	9	
Caroverine	6.6	P388/VCR	VCR (20)	19	Tsuruo, 1982
		P388/ADM	DOX (40)	8	
		K562/VCR	VCR (17)	11	Tsuruo, 1983a
Prenylamine	6.6	P388/VCR	VCR (20)	22	Tsuruo, 1982
		P388/ADM	DOX (40)	11	
		K562/VCR	VCR (17)	40	Tsuruo, 1983a
Bepiridil	4	CH <sup>R</sup> C5	DOX (195)	74	Schuurhuis, 1987
		2780 <sup>AD</sup>	DOX (140)	10	Schuurhuis, 1987

*Abbreviations:* ActD, actinomycin D; CH, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine.



TABLE 1-3

Summary of Studies Using Calmodulin Antagonists to  
Reverse Multidrug Resistance

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Trifluoperazine	3.3	P388/VCR	VCR (20)	10	Tsuruo, 1982
		P388/ADM	DOX (43)	5	
	2	P388/ADR	DOX (51)	2	Klohs, 1986
	4	P388/DOX	DOX (100)	5	Ganapathi, 1983b
	4	P388/DOX	DOX (100)	10	Ganapathi, 1984a
	5	P388/DOX	DOX (100)	10	Ganapathi, 1984a
			DAU (100)	15	
	2	P388/DOX	VLB (20)	5	Ganapathi, 1986
			VCR (100)	7	
	3.3	P388/ADM	DOX (27)	5	Tsuruo, 1983
		P388/VCR	VCR (26)	10	
		K562/VCR	VCR (17)	25	
	6.6	K562/VCR	VCR (17)	25	Tsuruo, 1983a
Thioridazine	5	KB-Ch <sup>R</sup> -24	CH (115)	58	Akiyama, 1986
			DOX (50)	19	
			DAU (36)	15	
			VLB (40)	40	
			VCR (68)	12	
	5	L1210/0.025	DOX (5)	3	Ganapathi, 1988
		L1210/0.05	DOX (10)	4	
		L1210/0.1	DOX (20)	7	
		L1210/0.25	DOX (40)	8	
	1	P388/ADR	DOX (50)	3	Ramu, 1984c
	4	KB-Ch <sup>R</sup> -24	CH (115)	14	Akiyama, 1986
			DOX (50)	30	
			DAU (36)	28	
			VLB (40)	44	
			VCR (68)	15	

(Continued)



**TABLE 1-3**  
(Continued)

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Chlorpromazine	4	P388/DOX	DOX (100)	3	Ganapathi, 1984b
	10	CEM/VLB <sub>100</sub>	VLB (420)	11	Zamora, 1988
	5	KB-Ch <sup>R</sup> -24	CH (115)	4	Akiyama, 1986
			DOX (50)	9	
			DAU (36)	7	
			VLB (40)	4	
			VCR (68)	2	
Prochlorperazine	4	P388/DOX	DOX (100)	5	Ganapathi, 1984b
Clomipramine	10	P388/VCR	VCR (20)	22	Tsuruo, 1982
		P388/ADM	DOX (43)	4	
	6.6	K562/VCR	VCR (17)	14	Tsuruo, 1983a
	6.6	P388/VCR	VCR (26)	12	Tsuruo, 1983
		P388/ADM	DOX (27)	4	
		K562/VCR	VCR (17)	14	
W12	20	P388/DOX	DOX (100)	1	Ganapathi, 1984b
W13	20	P388/DOX	DOX (100)	2	Ganapathi, 1984b

*Abbreviations:* CH, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine.





TABLE 1-4

Summary of Studies Using Anthracycline and *Vinca* Alkaloid Analogs,  
Lysosomotropic Agents, and Other Drugs to Reverse Multidrug Resistance

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
ID-8279	2	P388/VCR	VCR (50) DAU (10)	50 5	Inaba, 1984
Vindoline	10 $\mu$ g/ml	P388/VCR	VCR (50) VLB (20) DOX (10) DAU (10)	50 50 10 10	Inaba, 1986
		P388/DOX	DOX (200) DAU (200)	100 100	
	50	CEM/VLB <sub>100</sub>	VLB (420)	>42	Zamora, 1988
	10	CEM/VLB <sub>1K</sub>	VLB (930)	13	Beck, 1988
Chloroquine	10	P388/ADR	DOX (50)	2	Ramu, 1984c
	2 $\mu$ g/ml	KB-Ch <sup>R</sup> -24	DOX (38) DAU (18) VCR (139) VLB (24) ActD (24)	4 3 3 2 4	Shiraishi, 1986
	50	CEM/VLB <sub>100</sub>	VLB (157) VCR (600) DOX (112) DAU (124)	13 10 3 4	Zamora, 1986
	50	CEM/VLB <sub>100</sub>	VLB (420)	10	Zamora, 1988
	50	CEM/VLB <sub>1K</sub>	VLB (930)	10	Beck, 1988]
Quinacrine	0.5 $\mu$ g/ml	P388/VCR P388/ADR	VCR (15) DOX (240)	15 5	Inaba, 1988
	5	CEM/VLB <sub>100</sub>	VLB (420)	12	Zamora, 1988
	50	CEM/VLB <sub>1K</sub>	VLB (930)	10	Beck, 1988
Quinidine	10	P388/VCR K562/VCR P388/ADM	VCR (16) VCR (65) DOX (41)	82 50 8	Tsuruo, 1984
Quinine	100	CEM/VLB <sub>100</sub>	VLB (420)	>32	Zamora, 1988

(Continued)



**TABLE 1-4**  
(Continued)

Chemosensitizer	[ $\mu$ M]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Perhexiline Maleate	10	P388/ADR	DOX (20) VLB (25)	12 14	Ramu, 1984a
	10	MCF-7 <sup>Ad</sup>	DOX (12) VLB (500)	3 9	Foster, 1988
Tamoxifen	3	P388/ADR	DOX (32)	12	Ramu, 1984b
	10	MCF-7 <sup>Ad</sup>	DOX (12) VLB (500)	3 8	Foster, 1988
Triparanol	3	P388/ADR	DOX (32)	10	Ramu, 1984b
Reserpine	5	CEM/VLB <sub>100</sub>	VLB (420)	>42	Zamora, 1988
	5	CEM/VLB <sub>1K</sub>	VLB (930)	120	Beck, 1988
Cepharanthine	2 $\mu$ g/ml	KB-Ch <sup>R</sup> -24	DOX (117) DAU (17) VCR (105) ActD (43)	25 16 87 39	Shiraishi, 1987
Amiodarone	4	DHD/K12/TR	DOX (NR)	32	Chauffert, 1986
Dipyridamole	10	P388/ADR	DOX (50)	15	Ramu, 1984c
Aclacinomycin A	5 $\mu$ g/ml	Friend Leukemia	DOX (1000) DAU (1000)	2 3	Tapiero, 1988

*Abbreviations:* ActD, actinomycin D; Ch, colchicine; DOX, ADR, ADM, doxorubicin; DAU, daunomycin; VCR, vincristine; VLB, vinblastine.



TABLE 1-5

## Summary of Studies Using Cyclosporines to Reverse Multidrug Resistance

Chemosensitizer	[ $\mu\text{g/ml}$ ]	Cell Line	Cytotoxic Drug (Fold Resistance)	Fold Reversal	Reference
Cyclosporin A	13.2	EA/DAU	DAU (2)	3	Slater, 1986b
	13.2	GM3639/L <sub>100</sub>	VCR (60) DAU (5)	50 4	Slater, 1986a
	10 $\mu\text{M}$	Hepatoma 129	DAU (NR)	2	Meador, 1987
	5	NCI-H69/LX4	DOX (60) VCR (1200)	25 200	Twentyman, 1987
	2	NCI-H69/LX4	DOX (100) VCR (150)	9 32	Twentyman, 1988
	1	CH <sup>R</sup> C5 Aux B1 (S)	DOX (90) DOX (1)	90 10	Chambers, 1988
	1	P388/DOX	DOX (100)	8	Hait, 1989
Cyclosporin C	5	NCI-H69/LX4	DOX (60)	8	Twentyman, 1987
Cyclosporin G	5	NCI-H69/LX4	DOX (60)	90	Twentyman, 1987
Cyclosporin H	5	NCI-H69/LX4	DOX (60)	2	Twentyman, 1987
32W8-032	2	NCI-H69/LX4	DOX (100) VCR (150)	9 167	Twentyman, 1988
B3-243	2	NCI-H69/LX4	DOX (100) VCR (150)	55 50	Twentyman, 1988
11-MeLeu CsA	1	CH <sup>R</sup> C5 Aux B1 (S)	DOX (90) DOX (1)	8 10	Chambers, 1988
11-MeLeu CsA	1	P388/DOX	DOX (100)	8	Hait, 1989

*Abbreviations:* CH, colchicine; DOX, doxorubicin; DAU, daunomycin; EA, Ehrlich ascites; S, sensitive; VCR, vincristine.



TABLE 1-6

Summary of *In Vivo* Studies to Reverse Multidrug Resistance

Chemosensitizer	Tumor	Dose (mg/kg)		Schedule	ILS (+%)	Reference
		CS	Drug			
Verapamil	P388/VCR	0	VCR	qd x 10	0	Tsuruo, 1981
		0	0.2		0	
		50	0.1		34	
		50	0.2		29	
		100	0.03		29	
Verapamil	EA/DR	100	0.1	qd x 5	45	Slater, 1982
		0	DAU		8	
		25	0.4		120	
		50	0.4		106	
Verapamil	P388/DOX	0	DOX	qd x 8	2	Radel, 1988
		0	1.0		7	
		75	1.0		25	
Verapamil	Sarcoma 180	0	BOU	qd x 4	0	Chitnis, 1985
		10	0.0		5	
		10	0.6		45	
Verapamil	P388/VCR	100	VCR	qd x 10	45	Tsuruo, 1983
	P388/ADM	125	ADM		37	
SDB-ethylene-diamine	P388/VCR	0	VCR	qd x 8	10	Yamaguchi, 1986
		0	0.1		22	
		40	0.0		12	
		40	0.03		48	
		40	0.1		44	
DMDP	P388/DOX	0	DOX	qh x 4 (DMDP)	16	Radel, 1988
		60	0	qd x 1 (DOX)	4	
		60	10		-36	
Diltiazem	P388/ADM	100	DOX	qd x 10	27	Tsuruo, 1983b
		125	1.0		40	
Diltiazem	P388/VCR	125	VCR	qd x 10	32	Tsuruo, 1983
	P388/ADM	125	ADM		40	
Nicardapine	P388/ADM	75	DOX	qd x 10	43	Tsuruo, 1983b
Nicardapine	P388/VCR	100	VCR	qd x 10	51	Tsuruo, 1983
	P388/ADM	125	ADM		43	
Niludapine	P388/ADM	75	DOX	qd x 10	33	Tsuruo, 1983b
Niludapine	P388/VCR	100	VCR	qd x 10	50	Tsuruo, 1983
	P388/ADM	125	ADM		33	

(Continued)





**TABLE 1-6**  
(Continued)

Chemosensitizer	Tumor	Dose (mg/kg)			Schedule	ILS (+%)	Reference
		CS	Drug				
Clomipramine	P388/VCR	100	VCR	0.1	qd x 10	31	Tsuruo, 1983
	P388/ADM	125	ADM	1.0		8	
<i>N</i> -Acetyl- Daunorubicin	EA/DR	0	DAU	1.5	qd x 4	0	Skovsgaard, 1980
		30		0.0		0	
		30		1.5		53	
Quinidine	P388/VCR	0	VCR	0.0	qd x 10	0	Tsuruo, 1984
		0		0.2		19	
		125		0.0		2	
		125		0.2		41	
Quinacrine	P388/VCR	0	VCR	0.0	qd x 5	0	Inaba, 1988
		0		0.1		30	
		40		0.0		29	
		40		0.1		85	
Cyclosporine A	EA/DR	0	DAU	0.0	qd x 5	0	Slater, 1986b
		0		0.3		11	
		80		0.3		>215	
		12.5		0.3		183	
		5		0.3		169	
Cyclosporine	Hep 129	0	DAU	0.0	qd x 2	0	Meador, 1987
		0		0.4		26	
		10		0.0		10	
		10		0.4		69	

*Abbreviations:* BOU, bouvadin; ; DOX, ADM, doxorubicin; DAU, daunomycin; EA/DR, Ehrlich ascites/daunomycin resistant; VCR, vincristine.



## Chapter II

# STRUCTURE ACTIVITY RELATIONSHIPS OF PHENOTHIAZINES AND RELATED DRUGS FOR INHIBITION OF CELL GROWTH AND REVERSAL OF MULTIDRUG RESISTANCE

### A. Introduction

The recent demonstration and elucidation of the phenomenon of MDR has led to the search for drugs that could sensitize highly resistant cancer cells to chemotherapeutic agents. As reviewed in Chapter I (Section D.2), several PTZs have been shown to be among the group of drugs known to modify MDR. As a class of chemosensitizers, the PTZs present a unique opportunity to study the importance of molecular structure on the antagonism of MDR, since a large number of PTZs and structurally related compounds have been synthesized but have not been studied as MDR chemosensitizers.

PTZs are also known to inhibit the activity of several cellular enzymes (167, 193, 195) and block the function of critical cellular receptors, such as those for dopamine (47). Among these cellular targets is calmodulin, a multifunctional calcium-binding protein (137) that has been implicated in the regulation of numerous cellular events, including that of normal and abnormal cellular proliferation (86, 182, 238). Consistent with these observations was the demonstration that PTZs and other CaM antagonists possess antiproliferative and cytotoxic effects (113) in proportion to their anti-CaM activity (84, 87, 136, 239). Although the mechanism by which PTZs and other drugs modulate MDR is not clear, it has been suggested that their pharmacological properties may be mediated by the calcium messenger system, since the active compounds are known to inhibit voltage-dependent calcium channels (62), CaM (241), and protein kinase C (200).

To elucidate the structural features required for the activity of PTZs and related compounds against cellular proliferation and MDR, the effects of a series of drugs with single molecular alterations have been studied for their ability to inhibit cell growth and to



sensitize the human MDR breast cancer cell line MCF-7/DOX to doxorubicin. Furthermore, to clarify the role of CaM in these processes, the anti-CaM effects of these compounds have been compared to their effects on cellular proliferation and MDR.

## **B. Materials and Methods**

### ***1. Cell Lines and Culture***

MCF-7 human breast cancer cells, and the multidrug resistant subclone MCF-7/DOX (kindly supplied by Dr. Ken Cowan, National Cancer Institute, Bethesda, Maryland), selected by stepwise exposure of parental cells to increasing concentrations doxorubicin, were maintained in exponential growth in Corning 75 cm<sup>2</sup> tissue culture flasks in RPMI 1640 medium supplemented with 5% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. MCF-7/DOX cells were approximately 200-fold more resistant to doxorubicin than the parental cell line, and maintained a stable MDR phenotype while grown in drug-free medium for a period of at least 3 months, after which they were discarded. Cell lines were routinely tested and found to be free of contamination by mycoplasma or fungi.

### ***2. Effect of Drugs on Cellular Proliferation and Multidrug Resistance***

Cells in exponential growth were digested with 0.5% trypsin (Gibco, Grand Island, New York) in 10 mM phosphate-buffered saline (PBS), disaggregated into single cell suspensions, counted electronically (Coulter, Hialeah, FL), and dispensed in 100 µl volumes into 96-well microtiter plates with a multichannel pipet (Flow Labs, Titertek) at a concentration of  $0.5 \times 10^4$  sensitive, or  $1.0 \times 10^4$  resistant cells per well. Cells were allowed to attach to the plastic and to resume growth for 24 hours prior to the addition of 100 µl of drug-containing medium. Drugs were dissolved in small amounts of sterile water or 1% DMSO (final culture concentration < 0.05% DMSO) before dilution with medium. Controls were exposed to vehicle-containing medium. Following a 48 hour incubation at 37° C, the cellular supernatants of each well were gently aspirated, and cells were fixed and stained with 100 µl of 0.5% methylene blue (Sigma) in 50% ethanol (w/v) for 30 minutes at



room temperature, as previously described (60). Unbound stain was removed by decanting, followed by emersion in three, 1-liter washes of distilled, deionized water. The plates were dried for 12 hours and the stained protein was solubilized with 200  $\mu$ l of sodium N-lauroyl sarcosine (Fluka, Switzerland) solution (1% v/v in PBS). The optical density of each well was determined by absorbance spectrophotometry at a wavelength of 600 nm with a microculture plate reader (Titertek Model MCC/340) interfaced to an Apple IIe computer. Inhibition of cell growth was expressed as a percentage of absorbance of vehicle-treated control cultures.

To determine the optimal conditions for this assay, plates were inoculated in duplicate with various initial cell concentrations. One half plate was assayed daily for five consecutive days by standard trypsin digestion and electronic cell counting, while the other half of each plate was stained as described above. Figure 2-1 demonstrates the linear correlation between  $A_{600}$  from stained wells and actual cell number for the MCF-7/DOX cell line with final cell concentrations between 0 and 50,000 cells per well. Similar results were obtained for the sensitive cell line. Final assay conditions were chosen to ensure that optical density measurements fell on the linear portion of this curve. This system has proven extremely reproducible, with less than 5% variability between  $IC_{50}$  values from dose-response curves from different experiments run on different days.

The effect of PTZs on cellular proliferation was examined by exposing cells grown in the dark to 0 - 100  $\mu$ M drug as described above with each condition repeated in quadruplicate.  $IC_{50}$  was the concentration of drug that reduced staining ( $A_{600}$ ) to 50% of vehicle-treated controls. Final  $IC_{50}$  values represent the average of 3 to 5 separate experiments.

The effect of PTZs on MDR was studied by exposing cells to 0 - 100  $\mu$ M doxorubicin in the absence or presence of a concentration of PTZ derivative that alone produced 10% inhibition of growth ( $IC_{10}$ ) as determined by 3 to 5 experiments run in quadruplicate. Dose-response curves were corrected for the 10% inhibition of cell growth caused by the





PTZs alone. The MDR Ratio was defined as the ratio of the IC<sub>50</sub> for doxorubicin alone divided by the IC<sub>50</sub> for doxorubicin in the presence of modifier. This ratio represents the increase in apparent potency of doxorubicin produced by each PTZ derivative.

$$\text{MDR Ratio} = \frac{\text{IC}_{50} \text{ Doxorubicin Alone}}{\text{IC}_{50} \text{ Doxorubicin} + \text{Chemosensitizer}}$$

### 3. Drugs

Doxorubicin was obtained from Sigma Chemical Co. (St. Louis, MO) and was freshly prepared in distilled water for each experiment. PTZ derivatives and related drugs were generously donated as follows: chlorpromazine hydrochloride, trifluoperazine dihydrochloride, chlorpromazine sulfoxide hydrochloride, 2-chloro-10-[2-(dimethylamino)ethyl]phenothiazine hydrochloride, 2-chloro-10-[4-(dimethylamino)butyl]phenothiazine hydrochloride, promazine hydrochloride, trifluopromazine hydrochloride, 2-thiomethylpromazine hydrochloride, 1-chloropromazine hydrochloride, 3-chloropromazine hydrochloride, 4-chloropromazine hydrochloride and prochlorperazine ethanedisulfonate by Dr. Charles Zirkle of Smith Kline and French Laboratories (Philadelphia, PA); 7-hydroxychlorpromazine, 3,8-dihydroxychlorpromazine, 7,8-dihydroxychlorpromazine, desmethylchlorpromazine hydrochloride and didesmethylchlorpromazine hydrochloride by Dr. Albert Manian of the National Institute of Mental Health (Bethesda, MD); promethazine hydrochloride by Wyeth Laboratories (Radnor, PA); chlorproethazine hydrochloride by Rhone-Poulenc (Paris, France); imipramine hydrochloride and 2-chloroimipramine hydrochloride by Geigy Pharmaceuticals (Summit, NJ); haloperidol, pimozide, penfluridol and 4-(4-chloro- $\alpha,\alpha,\alpha$ -trifluoro-*m*-tolyl)-1-[4,4-bis(*p*-fluorophenyl)butyl]-4-piperidinol (R-6033) by Dr. Pierre Laduron of Janssen Pharmaceutica (Beerse, Belgium); quinacrine dihydrochloride by Sterling-Winthrop Research Institute (Rensselaer, NY); fluphenazine by Dr. S. J. Lucania of E. R. Squibb and Sons. The following thioxanthene derivatives were generously supplied by Dr. John Hyttel of H. Lundbeck (Copenhagen, Denmark): *cis*- and *trans*-flupenthixol, *cis*- and *trans*-clopenthixol, *cis*- and *trans*-chlorprothixene, N 751, N 753 A, N 753 B, N 762 A, N 762 B, N 768 A, N 768 B, N 789, N 796 A, N 796 B,



and N 7006. *N*-(4-amino-hexyl)-5-chloronaphthalenesulphonamide (W7) and its derivatives 5-iodo-1-C<sub>6</sub>, 5-iodo-1-C<sub>8</sub>, 5-iodo-1-C<sub>12</sub>, and 5-cyano-1-C<sub>8</sub> were kindly supplied by Dr. Shiela MacNeil (Northern General Hospital, Sheffield, UK). CGS 9343B was a gift of Dr. Jon Norman (CIBA-GEIGY, Summit, NJ). 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H-7), perphenazine and amitriptyline were obtained from Sigma. Other reagents were of analytical grade and were obtained from general commercial sources.

#### 4. Statistical Analysis

Statistical analysis of each dose-response curve was performed by the method of Finney (61). Accordingly, IC<sub>50</sub>'s  $\pm$  standard errors for the inhibition of cellular proliferation by drugs alone or in combination were determined by linear regression analysis of the logit transformed data. The significance of each MDR Ratio was then determined using Student's two-tailed *t* test, and was expressed in terms of P values. 95% confidence intervals for each MDR Ratio were calculated by Fieller's Ratio of Means as modified by Bliss (25). Student's *t* test was also employed to analyze the significance of differences between MDR Ratios or IC<sub>50</sub>'s for inhibition of cellular proliferation by various drugs.

### C. Results

#### 1. Effect of Modifying the Phenothiazine Nucleus on Cellular Proliferation and Multidrug Resistance

Table 2-1 shows the structures, IC<sub>50</sub> values for inhibition of cell growth and MDR Ratios for a series of promazine derivatives having different substitutions on the PTZ nucleus. The unsubstituted PTZ, promazine, inhibited cell growth (IC<sub>50</sub> = 26  $\mu$ M) and sensitized MCF-7/DOX cells to doxorubicin by 20% (MDR Ratio = 1.2, *P* < 0.05 as compared to doxorubicin alone). Adding a chlorine at positions 1, 2, 3, or 4 increased potency against cell growth by up to 3-fold, with the most potent compound (chlorpromazine) having a chlorine at position 2 (IC<sub>50</sub> = 8  $\mu$ M). This latter compound also had the greatest effect against MDR, sensitizing resistant cells to doxorubicin by 60% (MDR



Ratio = 1.6,  $P < 0.001$  as compared to doxorubicin alone). Similarly, adding a  $-CF_3$  group at position 2 increased potency against cell growth and MDR. Accordingly, trifluopromazine ( $IC_{50} = 16 \mu M$ ) was a 1.6-fold more potent cytostatic agent than promazine ( $P < 0.001$ ), and was 67% more potent ( $P < 0.001$ ) against MDR (MDR Ratio = 2.0).

Conversely, adding a  $-OH$  to the PTZ nucleus decreased the potency against both processes. For example, 7-hydroxychlorpromazine was 2-fold less potent than promazine in inhibiting cell growth ( $P < 0.001$ ), while the dihydroxylated analogs, 7,8- and 3,8-dihydroxychlorpromazine, were up to 15-fold less potent than promazine as inhibitors of cellular proliferation ( $P < 0.001$ ), having  $IC_{50}$ 's of  $63 \mu M$  and  $400 \mu M$ , respectively. In addition, the hydroxylated analogs had no significant chemosensitizing activity, and further increased resistance to doxorubicin (MDR Ratios  $\leq 1.0$ ). Oxidation of the bridge sulfur (chlorpromazine sulfoxide) markedly reduced antiproliferative activity ( $IC_{50} = 500 \mu M$ ), but increased activity against MDR (MDR Ratio = 2.2).

## ***2. Influence of the Side Chain Amino Group on Cellular Proliferation and Multidrug Resistance***

Table 2-2 shows that PTZs containing tertiary amines (chlorpromazine and chlorproethazine), secondary amines (desmethylchlorpromazine), and primary amines (didesmethylchlorpromazine) possess similar activity against cellular proliferation ( $IC_{50}$ 's = 8 - 12  $\mu M$ ). However, PTZs having tertiary amines were more potent antagonists of MDR than those with secondary or primary amines, producing a 1.6- to 2.2-fold increase in sensitivity to doxorubicin. For example, the  $IC_{50}$ 's of chlorpromazine and desmethylchlorpromazine were equal (8  $\mu M$ ), whereas chlorpromazine was more potent than desmethylchlorpromazine against MDR (MDR Ratios = 1.6 versus 1.2,  $P < 0.01$ ). Other changes in the type of amino group also affected anti-MDR activity. For example, piperazinyl derivatives increased potency against MDR. Accordingly, the MDR Ratios for



trifluoperazine (3.4) and fluphenazine (2.7), compounds with piperazinyl amino side chains, were greater than that of trifluopromazine (MDR Ratio = 2.0,  $P < 0.001$  and 0.001, respectively), a compound with an identical hydrophobic ring-substitution, but possessing an aliphatic side chain. Similarly, perphenazine and prochlorperazine (MDR Ratios = 2.0 and 2.6) were more potent antagonists of MDR than chlorpromazine (MDR Ratio = 1.6,  $P < 0.01$  and 0.001, respectively). This series also points out the importance of the  $-CF_3$  substitution at position 2 for anti-MDR activity. For example, the MDR Ratio for trifluoperazine (3.4) was greater than that of prochlorperazine (MDR Ratio = 2.6,  $P < 0.01$ ). These PTZs have identical structures except that the former has a  $-CF_3$  instead of a  $-Cl$  at position 2. A similar relationship is seen by comparing the MDR Ratio for fluphenazine (2.7) to perphenazine (MDR Ratio = 2.0,  $P < 0.01$ ), also identical molecules except for the  $-CF_3$  ring substitution. Finally, a para-methyl substitution on the piperazine appeared more potent than an ethanol group for anti-MDR activity of compounds, as seen by comparing the MDR Ratios for prochlorperazine (2.6) to perphenazine (2.0,  $P < 0.05$ ), or trifluoperazine (3.4) to fluphenazine (2.7,  $P < 0.001$ ).

### *3. Influence of Alkyl Bridge Length on Cellular Proliferation and Multidrug Resistance*

Table 2-3 shows the effect on cell growth and on MDR of a series of 10-[alkyl-dimethylamino]phenothiazines in which the length of the alkyl bridge connecting the PTZ nucleus to the amino group was varied. Increasing the distance between the ring nucleus and the amino group from two to four carbons increased the antiproliferative and chemosensitizing effects of these compounds. For example, 2-chloro-10-[4-(dimethylamino)butyl]phenothiazine, which has a four carbon alkyl bridge, was a more potent antiproliferative agent ( $IC_{50} = 7 \mu M$ ) and anti-MDR agent (MDR Ratio = 2.0) than any of the other four compounds with two or three carbon alkyl chains. Conversely, promethazine, which has an isopropyl side chain was a less potent inhibitor of cell growth than promazine, which has a three carbon chain.





#### ***4. Influence of Hydrophobicity of Phenothiazines on Cellular Proliferation and Multidrug Resistance***

To determine the influence of hydrophobicity on the effect of PTZs on cellular proliferation and MDR, the octanol:buffer partition coefficients were compared for each of the 10 ring-substituted promazine derivatives as previously determined (174), to the IC<sub>50</sub>'s for inhibition of cell growth and to the MDR Ratios. Figures 2-2 A and B demonstrate the excellent correlation between hydrophobicity and both antiproliferative activity ( $r = -0.73$ ,  $P = 0.016$ ) and MDR antagonism ( $r = 0.86$ ,  $P = 0.0015$ ).

To determine if the differences in potency of compounds with side chain alterations were also due to changes in overall hydrophobicity, we compared the octanol:buffer partition coefficients for each of the drugs in Tables 2-2 and 2-3 that had -Cl substitutions at position 2 of the PTZ ring to their IC<sub>50</sub>'s for inhibition of cell growth and to their MDR Ratios (Figures 2-2 C and D). In contrast to the results for ring-substituted PTZs, no statistically significant correlation was found between hydrophobicity and potency of compounds with side chain alterations for inhibition of cell growth ( $r = 0.54$ ,  $P = 0.27$ ) or antagonism of MDR ( $r = 0.59$ ,  $P = 0.21$ ).

#### ***5. Correlation Between Anti-Calmodulin Activity and Inhibition of Cellular Proliferation and Multidrug Resistance***

To examine the role of CaM as a possible target for the effect of PTZs on cellular proliferation and MDR, the IC<sub>50</sub>'s for the inhibition of CaM by PTZs and structurally related compounds (174) were compared to their IC<sub>50</sub>'s for the inhibition of cell growth, and to their effect on MDR when used at equimolar (3  $\mu$ M) concentrations. Figures 2-3 A and B show a good correlation between anti-CaM activity and antiproliferative activity ( $r = 0.58$ ,  $P = 0.0009$ ), whereas no correlation was found between anti-CaM activity and chemosensitizing activity ( $r = 0.17$ ,  $P = 0.44$ ). Similar results were found for equitoxic concentrations of PTZs.



## *6. Effect of Phenothiazines and Structurally Related Compounds on Doxorubicin-Sensitive Cells*

The activity of all PTZs and structurally related compounds against cell growth and as modulators of sensitivity to doxorubicin was examined against the doxorubicin-sensitive MCF-7 cell line. Each of the drugs tested were equally potent antiproliferative agents against the doxorubicin-sensitive cell line as compared to their activity against the resistant MCF-7/DOX cell line (data not shown). No compound sensitized the MCF-7 cells to doxorubicin.

## *7. Effect of Specific Inhibitors of Calmodulin and Protein Kinase C on Cell Growth and Multidrug Resistance*

Table 2-4 shows the reported values for inhibition of CaM and PKC by several "specific" inhibitors of each, and their effect on cell growth and MDR, respectively. While a wide range of anti-CaM activities are reported for the W7 derivatives, these compounds had approximately similar antiproliferative effects (18 - 22  $\mu\text{M}$ ), and showed only little or no chemosensitizing activity (MDR Ratios = 1.0 to 1.5). The relatively specific CaM antagonist CGS 9343B displayed slightly greater chemosensitizing effects, causing a 2-fold reversal of doxorubicin resistance when used at the non-toxic concentration of 6  $\mu\text{M}$ . H-7, a competitive inhibitor of PKC, was an extremely non-toxic substance, showing significant antiproliferative effects only at concentrations  $\geq 200 \mu\text{M}$ . Therefore, much higher  $\text{IC}_{10}$  doses were used than for most of the PTZs (30  $\mu\text{M}$ ). Nevertheless, H-7 had no anti-MDR activity, and actually caused a significant, though slight, enhancement of doxorubicin resistance.

## *8. Effect of Compounds Structurally Related to the Phenothiazines on Inhibition of Cellular Proliferation and Multidrug Resistance*



Tables 2-5 and 2-6 show the structures, IC<sub>50</sub> values for inhibition of cell growth and effect on MDR for several classes of compounds that incorporate certain structural features identified as important for activity from the PTZ studies. Penfluridol and quinacrine (Table 2-5) were potent antiproliferative agents (IC<sub>50</sub> = 3  $\mu$ M), but were relatively poor antagonists of MDR (MDR Ratios = 2.0 and 1.3, respectively).

Table 2-6 shows that many of the thioxanthene isomers were particularly effective chemosensitizers. This class of compounds differ from the PTZs by a carbon substitution at position 10 of the PTZ nucleus, and an exocyclic double bond to the side chain.

Each of the active chemosensitizing thioxanthene isomers caused a significantly greater potentiation of doxorubicin activity than their respective PTZ homolog (Tables 2-1 to 2-3), as demonstrated by comparing the MDR Ratios for *trans*-chlorprothixene to chlorpromazine (7.0 versus 1.6,  $P < 0.001$ ), *trans*-768 to chlorproethazine (7.2 versus 2.2,  $P < 0.001$ ), *trans*-796 to trifluoperazine (2.8 versus 2.0,  $P < 0.01$ ), *trans*-clopenthixol to perphenazine (15.0 versus 2.0,  $P < 0.001$ ), and *trans*-flupenthixol to fluphenazine (15.2 versus 2.7,  $P < 0.001$ ).

In addition, Table 2-6 demonstrates that the thioxanthenes displayed stereospecificity in their effect on MDR, since the *trans*-isomer of each active pair of thioxanthenes was 2- to 6-fold more effective than the *cis*-isomer for the reversal of MDR at equitoxic concentrations. For example, Figure 2-4 displays representative dose-response curves of MCF-7/DOX cells to doxorubicin in the absence or presence of PTZ and thioxanthene isomers, and shows that *trans*-flupenthixol was a 3-fold more effective chemosensitizer than *cis*-flupenthixol, and 6-fold more effective than the PTZ homolog fluphenazine, at equitoxic doses. This relationship also was true for equimolar concentrations of PTZs and thioxanthenes (data not shown).

Similar to the PTZs, alterations in the length or type of side chain affected anti-MDR activity for the thioxanthenes. Aliphatic side chains of two carbons in length had little chemosensitizing activity (*cis*- or *trans*-762), while several thioxanthenes with three carbon aliphatic side chains possessed increased activity (*trans*-chlorprothixene, *trans*-



768). It was also necessary for cyclic ring side chains substituents to be a distance greater than two carbons from the thioxanthene ring nucleus for activity, as demonstrated by *trans*-7006 (MDR Ratio = 1.6) compared to *trans*-clopenthixol (MDR Ratio = 15.0), otherwise identical molecules except for a two carbon versus three carbon alkyl bridge.

The most effective thioxanthene chemosensitizers, *trans*-clopenthixol, *trans*-flupenthixol and *trans*-753, caused an approximately 15-fold reversal of MDR, and each possessed cyclic piperazinyl or piperadinyll substituents at a distance of three carbons from the thioxanthene ring, in a *trans* configuration. Slightly higher concentrations of *trans*-flupenthixol (8  $\mu$ M) sensitized MDR cells to doxorubicin by 35-fold, a 10-fold greater anti-MDR activity than equitoxic ( $IC_{10}$ ) doses of trifluoperazine, the most potent PTZ antagonist of MDR (data from Section III.C.6).

In contrast to the anti-MDR effects of these compounds, the thioxanthene isomers displayed a relative lack of potency and stereospecificity as antiproliferative agents. For example, *cis*- and *trans*-flupenthixol possessed only modest activity against cell growth ( $IC_{50}$ 's of 24 and 25  $\mu$ M), similar in potency to its PTZ homolog fluphenazine ( $IC_{50}$  = 23  $\mu$ M) and less potent than many of the other PTZs tested. Similar comparisons may be made for each of the thioxanthene isomers and their PTZ homologs.

## D. Discussion

The results presented in this chapter demonstrate that specific structural features of the PTZ molecule affect its activity against cellular proliferation and MDR, that the antagonism of CaM does not appear to be directly related to the PTZs' anti-MDR activity, and that the thioxanthene class of antipsychotic drugs are more effective chemosensitizers than the PTZs.

The data presented show that even slight modifications in the chemical structure of the PTZs can dramatically alter their potencies as antiproliferative and chemosensitizing agents, and suggest that these two actions of the PTZs are mediated by different mechanisms.





Specifically, increasing the hydrophobicity of the PTZ nucleus increased potency against cellular proliferation and against MDR, whereas decreasing the hydrophobicity decreased potency (Table 2-1). Thus, the  $-CF_3$  substituted compounds were the most potent drugs, whereas  $-OH$  substituted compounds were the least potent drugs. Chlorpromazine sulfoxide, the oxidative metabolite of chlorpromazine, lost most of its antiproliferative effect. However, it retained its effect against MDR, suggesting that first-pass hepatic metabolism of these drugs may not present a major impediment to the clinical use of PTZs as chemosensitizers.

The type of amino group also significantly affected potency against MDR but not against cellular proliferation. For example, tertiary amines were more potent than primary or secondary amines, and piperazinyl amines were more potent than non-cyclic groups. Moreover, piperazinyl structures that possessed a para-methyl group had consistently greater activity than others (Table 2-2).

The distance between the amino group and the PTZ nucleus was important for both inhibition of cell growth and antagonism of MDR. A four carbon chain was superior to alkyl bridges of shorter lengths (Table 2-3). Whether an alkyl bridge of greater than four carbons would further increase activity could not be determined in the present study since these derivatives were not available.

It has been postulated that the effects of the PTZs may be due solely to non-specific membrane interactions resulting from their high degree of lipophilicity (191). A careful analysis of the relationship between hydrophobicity and inhibition of cellular proliferation or antagonism of MDR showed a correlation for ring-substituted PTZ derivatives (Figures 2-2 A and B), but not for compounds with specific side chain alterations (Figures 2-2 C and D). Thus, the degree of lipophilicity of each drug, while important, was not the sole determinant of potency for the antiproliferative or anti-MDR activity of PTZs. In fact, specific structural features of the side chain also determine potency, such as the nature and distance between the amino groups and PTZ nucleus, suggesting electrostatic interactions between the positively-charged amine and a particular cellular target protein may also be important.



This profile is reminiscent of that displayed by the interaction of PTZs with CaM. Studies of the structural features of PTZs that influence CaM antagonism revealed that ring-substitutions that increased hydrophobicity increased potency, while modifications of the type or length of the amino side chain affected potency in a manner unrelated to hydrophobicity (174). Similarly, studies with *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W5) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) (97), and a series of 15 derivatives of W7 (143), demonstrated that both halogenation of the naphthalene ring with chlorine, iodine or cyano groups, and increasing the length of the alkyl side chain from 4 to 12 carbons increased their potency against CaM.

Drug binding studies with synthetic peptides and molecular modeling provided a rationale for the importance of both hydrophobicity and molecular structure for the PTZ - CaM interaction. The induction of  $\alpha$ -helix formation by the binding of  $\text{Ca}^{++}$  to CaM results in two distinct regions, a hydrophobic pocket containing two aromatic phenylalanine residues (Phe 89 and 92) oriented to form a charge transfer complex with the aromatic, tricyclic nucleus of the PTZs, and a hydrophilic region at a distance of one-half helical turn formed by glutamic acid residues (Glu 83, 84 and 87), which interact with the positively-charged nitrogen atom of the PTZ side chain (183). The relationship between structure and hydrophobicity of the PTZs and their antiproliferative and anti-MDR activities suggests that in these systems, similar to CaM, the PTZs interact in both a hydrophobic and electrostatic manner with a protein target. Like CaM, it is likely that this target possesses a hydrophobic domain in close proximity to a negatively-charged amino acid.

The site(s) of action of PTZs and structurally related compounds for inhibition of cellular proliferation and antagonism of MDR have not been identified. The current data demonstrate that the antiproliferative activity of these drugs used individually against the malignant breast cancer cell lines MCF-7 and MCF-7/DOX correlate with their potency as CaM antagonists (Figure 2-3 A), supporting and extending previous observations with a limited number of PTZs in MDA-MB-231 human breast cancer cells (239), C<sub>6</sub> astrocytoma cells (136), HL-60 human leukemia cells, L1210 murine leukemia cells, and HCT-8 human



colonic carcinoma cells (84). These data are consistent with the known role of CaM in cellular proliferation (86, 182), and suggest a role for CaM as the PTZ target involved in cell growth inhibition by PTZs. However, the complete lack of correlation between anti-CaM activity and antagonism of MDR by equimolar (Figure 2-3 B), as well as equitoxic (data not shown) concentrations of PTZs, points toward an alternative mechanism of inhibition for this effect. This is in contrast to conclusions reached by Ganapathi (73), who compared the anti-CaM and anti-MDR activity of trifluoperazine, chlorpromazine, and prochlorperazine in murine P388/DOX cells. If one examines the present data for these 3 drugs alone, a correlation also is found between anti-CaM and anti-MDR activity (Figure 2-3B). However, in the context of the much larger sample size in the present study, this correlation does not remain significant.

Since the PTZs are considered non-specific probes for CaM function in cell systems due to their activity as inhibitors of PKC (200) and dopamine receptors (47), the chemosensitizing effect of several compounds with relatively increased specificity for CaM or PKC were examined. While several analogs of the naphthalene sulphonamide W7 possess far less PKC inhibitory activity, along with increased anti-CaM activity (143), these compounds caused little or no potentiation of doxorubicin toxicity in MCF-7/DOX cells (Table 2-5). Similarly, the CaM antagonist CGS 9343B, which also lacks potency as an inhibitor of PKC (160), was found to be a fairly poor chemosensitizer in MDR cells (Table 2-4). These data further support the previous evidence that antagonism of CaM is not directly involved in the reversal of MDR by chemosensitizers. However, it appears that inhibition of CaM may be a useful marker for potential MDR chemosensitizers, since the structural requirements for both are similar, and many of the presently studied chemosensitizers are also potent CaM antagonists.

Finally, the isoquinolinesulfonyl H-7, a potent and competitive inhibitor of PKC (98, 162), had no chemosensitizing effect on MDR cells, but slightly enhanced the resistance of cells to doxorubicin (Table 2-4), in agreement with preliminary results reported by



Ganapathi (70). It is interesting to note that Ido *et al* (105) found H-7 capable of inhibiting a TPA-induced decrease in vincristine accumulation in sensitive P388 cells. Therefore, it appears that while PKC activation by phorbol esters may induce MDR, the inhibition of PKC does not necessarily sensitize MDR cells.

The information gained from these studies enabled the identification of additional drugs with certain of the important structural features for chemosensitizing activity. A number of tricyclic compounds with cationic amino group side chains were studied for chemosensitizing activity. Several, such as imipramine and 2-chloroimipramine had modest anti-MDR activity. Interestingly, quinacrine, a compound described as sensitizing several other MDR cell lines (109, 246), showed essentially no anti-MDR activity in the present study, probably because a very low dose was used due to its potent antiproliferative effects ( $IC_{10} = 0.75 \mu M$ ).

The most interesting results were displayed by the thioxanthene class of antipsychotic drugs (Table 2-6). Nearly every *trans*-thioxanthene isomer possessed greater chemosensitizing activity than their respective PTZ homologs, and several, such as *trans*-flupenthixol, *trans*-clopenthixol, and *trans*-753, were 5- to 7-fold more effective at equitoxic or equimolar concentrations. Many of the structural features important for PTZ anti-MDR activity were also effective for thioxanthenes. For example, thioxanthenes with aliphatic or cyclic side chains of three carbons in length were more effective than those with two carbon alkyl bridges. Also, piperazinyl or other cyclic side group substitutions resulted in greater activity than aliphatic side groups. The most effective pair of thioxanthenes, *cis*- and *trans*-flupenthixol, have a  $-CF_3$  substitution at position 2 of the hydrophobic thioxanthene ring, possess a piperazinyl amino side chain, and have a 3 carbon alkyl bridge.

While the thioxanthene isomers are more hydrophobic than PTZs due to the substitution of a carbon for a nitrogen in the cyclic ring, this alone cannot explain their cellular effects. For example, the octanol:buffer partition coefficients ( $\log P$ ) for both flupenthixol isomers = 4.25 versus 4.04 for chlorpromazine (161), but they are less potent antiproliferative agents than chlorpromazine and other less hydrophobic PTZs. In addition, while the





isomers are equally hydrophobic, *trans*-flupenthixol is a 3-fold more potent anti-MDR agent (Figure 2-4), and both isomers are more potent than agents with greater hydrophobicity, such as pimozide ( $\log P = 4.88$ ).

The orientation of the side chain amine in relation to the tricyclic nucleus was an important determinant of anti-MDR activity, but not of antiproliferative activity for the thioxanthenes. For example, *trans*-flupenthixol displayed greater activity than the *cis*-flupenthixol against MDR (Figure 2-4), but was equal to that of the *cis*-isomer against cellular proliferation (Table 2-6). In fact, for every pair of active thioxanthene isomers, the *trans*-isomer was uniformly more effective than the *cis*- for reversing MDR, while no consistent stereoisomerism was seen for the antiproliferative effects of these compounds. It is intriguing that compound 789, which is identical to clopenthixol except for a single instead of a double bond connecting the piperazinyl side chain to the thioxanthene ring nucleus, was nearly as effective as *trans*-clopenthixol, and far more effective than *cis*-clopenthixol or perphenazine. This suggests that the superior anti-MDR activity of the thioxanthenes may be due to specific binding to a receptor with greater affinity for compounds in the *trans* configuration, or compounds which are able to assume the *trans* configuration, and that drugs locked into a *cis* orientation by a double bond are unable to bind with high affinity possibly due to steric hindrance.

Taking into account all of the principles derived from this analysis of PTZ-like compounds, structural features important for a drug to alter MDR include a hydrophobic thioxanthene ring nucleus with a  $-\text{CF}_3$  substitution at position 2, an exocyclic double bond in the *trans* configuration, and a piperazinyl amine with a para-methyl or ethanol group, joined by a 3 or 4 carbon alkyl bridge to the nucleus. These findings agree with similar structural features identified as important for modulating MDR in human leukemic cells in a recent study with derivatives of indole alkaloids (246). Therefore, the present structure-activity studies more clearly define the 'pharmacophore' necessary for specific anti-MDR activity for PTZs and structurally related classes of drugs, and have identified a previously undescribed class of chemosensitizers, which contain several lead compounds worthy of



further examination.

Clinical trials of the antipsychotic effects of the thioxanthene flupenthixol in humans showed that *cis*-flupenthixol was far more effective than *trans*-flupenthixol, and that the latter was far less toxic (116). This observation may be explained by biochemical and crystallographic evidence that *cis*-flupenthixol is a potent antagonist of dopamine receptors (103, 172), whereas *trans*-flupenthixol, which displays the greater potency against MDR, has virtually no activity as a dopamine antagonist. This may explain the apparent lack of extrapyramidal side effects seen with this agent (157). Extrapyramidal side effects have proven to be dose limiting in Phase I trials that combined trifluoperazine with bleomycin (88) or doxorubicin (149). Therefore, further *in vitro* and *in vivo* characterization of chemosensitizing activity of *cis*- and *trans*-flupenthixol were undertaken.



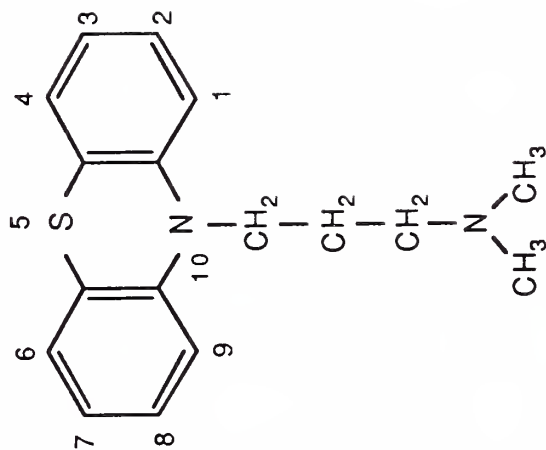


**TABLE 2-1**

**Effect of Modifying the Phenothiazine Nucleus on Activity Against Cell  
Growth and Multidrug Resistance**

Inhibition of cell growth was determined by exposing MCF-7/DOX cells to 0 - 100  $\mu$ M of each PTZ.  $IC_{50}$  is the mean concentration  $\pm$  S.E. that produced 50% inhibition of cell growth compared to vehicle-treated controls, as described in Materials and Methods (Section B.2). To determine the MDR Ratio, MCF-7/DOX cells were exposed to 0-100  $\mu$ M doxorubicin in the presence or absence of PTZ at a concentration that alone produced 10% inhibition of cellular growth. MDR Ratio is the  $IC_{50}$  for doxorubicin alone divided by the  $IC_{50}$  doxorubicin in the presence of PTZ. 95% confidence intervals and P- values for comparison of  $IC_{50}$ 's for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were derived as described in Methods. All values represent the mean of three to five separate experiments; each experiment was done in quadruplicate.

# Effect of Modifying the Phenothiazine Nucleus



Substituent	Position	Name	Cell Growth Inhibition $IC_{50} \pm S.E. (\mu M)$	MDR Ratio $\pm 95\% C.I.$
		Promazine		
-Cl	1	1-Chloropromazine	$26 \pm 4$	$1.2 \pm 0.2^a$
-Cl	2	Chlorpromazine	$21 \pm 5$	$1.3 \pm 0.2^b$
-Cl	3	3-Chloropromazine	$8 \pm 1$	$1.6 \pm 0.3^b$
-Cl	4	4-Chloropromazine	$10 \pm 2$	$1.3 \pm 0.2^b$
-Cl; -OH	2;7	7-Hydroxychlorpromazine	$15 \pm 3$	$1.4 \pm 0.3^b$
-Cl; -OH; -OH	2;3;8	3,8-Dihydroxychlorpromazine	$50 \pm 7$	$1.0 \pm 0.3^c$
-Cl; -OH; -OH	2;7;8	7,8-Dihydroxychlorpromazine	$400 \pm 45$	$0.9 \pm 0.3^c$
-S-CH <sub>3</sub>	2	Thiomethylpromazine	$63 \pm 22$	$0.8 \pm 0.2^a$
-CF <sub>3</sub>	2	Trifluopromazine	$20 \pm 3$	$1.5 \pm 0.2^b$
=O	5	Chlorpromazine Sulfoxide	$16 \pm 3$	$2.0 \pm 0.3^b$
			$500 \pm 95$	$2.2 \pm 0.4^b$

<sup>a</sup>  $P < 0.05$

<sup>b</sup>  $P < 0.001$

<sup>c</sup> P. not significant





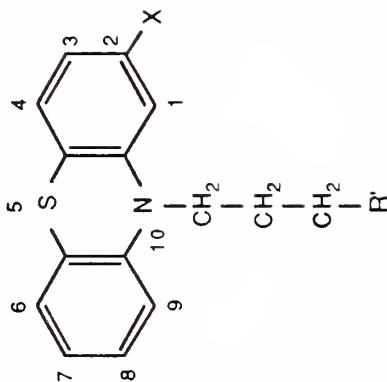


**TABLE 2-2**

**Effect of Modifying the Type of Side Chain Amino Group on Activity  
Against Cell Growth and Multidrug Resistance**

MCF-7/DOX cells were treated as described in Table 2-1.  $IC_{50}$  values  $\pm$  S.E. and MDR Ratios with 95% confidence intervals and P- values for comparison of  $IC_{50}$ 's for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 2-1. Each value represents the mean of three to five separate experiments; each experiment was done in quadruplicate.

## Effect of Modifying the Type of Side Chain Amino Group



X	R'	Name	Cell Growth Inhibition $IC_{50} \pm S.E. (\mu M)$	MDR Ratio $\pm 95\% C.I.$
—Cl	—NH <sub>2</sub>	Didesmethylchlorpromazine	11 $\pm$ 1	1.1 $\pm$ 0.1 <sup>a</sup>
—Cl	—NH—CH <sub>3</sub>	Desmethylchlorpromazine	8 $\pm$ 2	1.2 $\pm$ 0.2 <sup>b</sup>
—Cl	—N(CH <sub>3</sub> ) <sub>2</sub>	Chlorpromazine	8 $\pm$ 1	1.6 $\pm$ 0.3 <sup>a</sup>
—Cl	—N(CH <sub>2</sub> CH <sub>3</sub> ) <sub>2</sub>	Chlorproethazine	12 $\pm$ 1	2.2 $\pm$ 0.4 <sup>a</sup>
—Cl	—N(CH <sub>2</sub> CH <sub>2</sub> OH) <sub>2</sub>	Perphenazine	32 $\pm$ 4	2.0 $\pm$ 0.3 <sup>a</sup>
—Cl	—N(CH <sub>3</sub> ) <sub>2</sub>	Prochlorperazine	22 $\pm$ 3	2.6 $\pm$ 0.4 <sup>a</sup>
—CF <sub>3</sub>	—N(CH <sub>3</sub> ) <sub>2</sub>	Trifluopromazine	16 $\pm$ 3	2.0 $\pm$ 0.3 <sup>a</sup>
—CF <sub>3</sub>	—N(CH <sub>2</sub> CH <sub>2</sub> OH) <sub>2</sub>	Fluphenazine	23 $\pm$ 5	2.7 $\pm$ 0.3 <sup>a</sup>
—CF <sub>3</sub>	—N(CH <sub>3</sub> ) <sub>2</sub>	Trifluoperazine	19 $\pm$ 3	3.4 $\pm$ 0.4 <sup>a</sup>

<sup>a</sup>  $P < 0.001$ <sup>b</sup>  $P < 0.01$



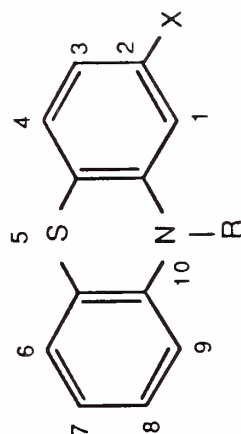


**TABLE 2-3**

**Effect of Modifying the Length of the Side Chain Amino Group on Activity  
Against Cell Growth and Multidrug Resistance**

MCF-7/DOX cells were treated as described in Table 2-1.  $IC_{50}$  values  $\pm$  S.E. and MDR Ratios with 95% confidence intervals and P- values for comparison of  $IC_{50}$ 's for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 2-1. Each value represents the mean of three to five separate experiments; each experiment was done in quadruplicate.

# Effect of Modifying the Length of the Side Chain Amino Group



X	R	Name	Cell Growth Inhibition IC <sub>50</sub> ± S.E. (μM)	MDR Ratio ± 95% C.I.
—Cl		2-Chloro-10-[2-(dimethylamino)ethyl] phenothiazine	27 ± 4	1.5 ± 0.3 <sup>a</sup>
—Cl		Chlorpromazine	8 ± 1	1.6 ± 0.3 <sup>a</sup>
—Cl		2-Chloro-10-[4-(dimethylamino)butyl] phenothiazine	7 ± 1	2.0 ± 0.3 <sup>a</sup>
—H		Promethazine	29 ± 5	1.9 ± 0.5 <sup>a</sup>
—H		Promazine	26 ± 4	1.2 ± 0.2 <sup>b</sup>

<sup>a</sup> P < 0.001

<sup>b</sup> P < 0.05





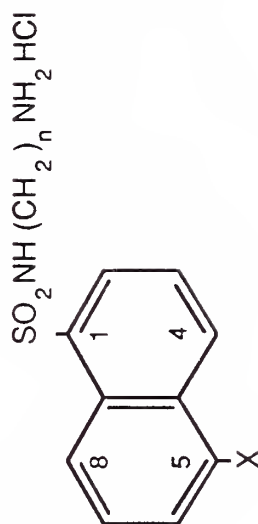


TABLE 2-4

**Effect of Specific Inhibitors of Calmodulin and Protein Kinase C  
on Cell Growth and Multidrug Resistance**

MCF-7/DOX cells were treated as described in Table 2-1.  $IC_{50}$  values  $\pm$  S.E. and MDR Ratios with 95% confidence intervals and P- values for comparison of  $IC_{50}$ 's for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 2-1. Each value represents the mean of two to three separate experiments; each experiment was done in quadruplicate. Values for inhibition of CaM and PKC are from MacNeil *et al* (1988) for W7 and derivatives, Norman *et al* (1987) for CGS 9343B, and Hidaka *et al* (1984) for H-7.

# Effect of Specific Calmodulin and Protein Kinase C Antagonists



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Name	X	n	CaM IC <sub>50</sub> (μM)	PKC IC <sub>50</sub> (μM)	Cell Growth Inhibition IC <sub>50</sub> ± S.E. (μM)	MDR Ratio ± 95% C.I.
5-chloro-1-C <sub>6</sub> (W7)	Cl	6	29	342	28 ± 4	1.0 ± 0.1 <sup>a</sup>
5-iodo-1-C <sub>6</sub>	I	6	29	>1000	18 ± 2	1.0 ± 0.1 <sup>a</sup>
5-iodo-1-C <sub>8</sub>	I	8	4	>1000	22 ± 1	1.0 ± 0.1 <sup>a</sup>
5-iodo-1-C <sub>12</sub>	I	12	0.7	ND	18 ± 2	1.4 ± 0.2 <sup>b</sup>
5-cyano-1-C <sub>8</sub>	CN	8	2	ND	18 ± 3	1.5 ± 0.2 <sup>b</sup>
CGS 9343B			3.3	100	26 ± 5	2.2 ± 0.4 <sup>a</sup>
H-7			97	6	>200	0.8 ± 0.1 <sup>b</sup>

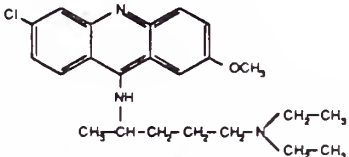
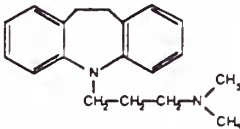
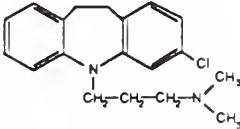
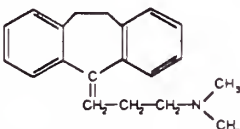
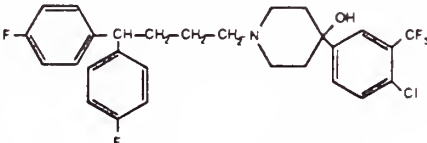
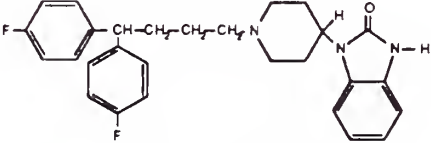
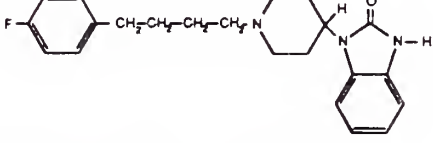
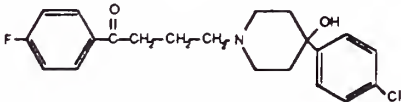
<sup>a</sup> P < 0.05

<sup>b</sup> P < 0.001



TABLE 2-5

**Effect of Compounds Structurally Related to the Phenothiazines  
on Cell Growth and Multidrug Resistance**

Compound	Structure	Cell Growth Inhibition	MDR Ratio
		IC <sub>50</sub> ± S.E. (μM)	± 95% C.I.
Quinacrine		3 ± .1	1.3 ± 0.1 <sup>b</sup>
Imipramine		19 ± 5	2.5 ± 0.9 <sup>a</sup>
2-Chloroimipramine		20 ± 2	2.0 ± 0.6 <sup>a</sup>
Amitriptyline		40 ± 5	1.3 ± 0.1 <sup>c</sup>
Pentfluridol		3 ± .2	2.0 ± 0.3 <sup>a</sup>
Pimozide		50 ± 20	1.3 ± 0.5 <sup>c</sup>
R-6033		27 ± 4	1.1 ± 0.2 <sup>d</sup>
Haloperidol		750 ± 300	3.3 ± .7 <sup>a</sup>

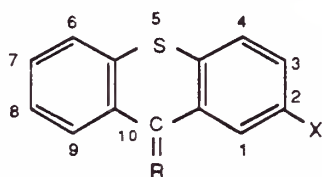
<sup>a</sup> P < 0.001<sup>b</sup> P < 0.05<sup>c</sup> P < 0.01<sup>d</sup> P - not significant

MCF-7/DOX cells were treated as described in Table 2-1. IC<sub>50</sub> values ± S.E. and MDR Ratios with 95% confidence intervals and P- values for comparison of IC<sub>50</sub>'s for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 2-1. Each value represents the mean of three to five separate experiments; each experiment was done in quadruplicate.



TABLE 2-6

**Effect of Thioxanthene Stereoisomers on Cell Growth  
and Multidrug Resistance**



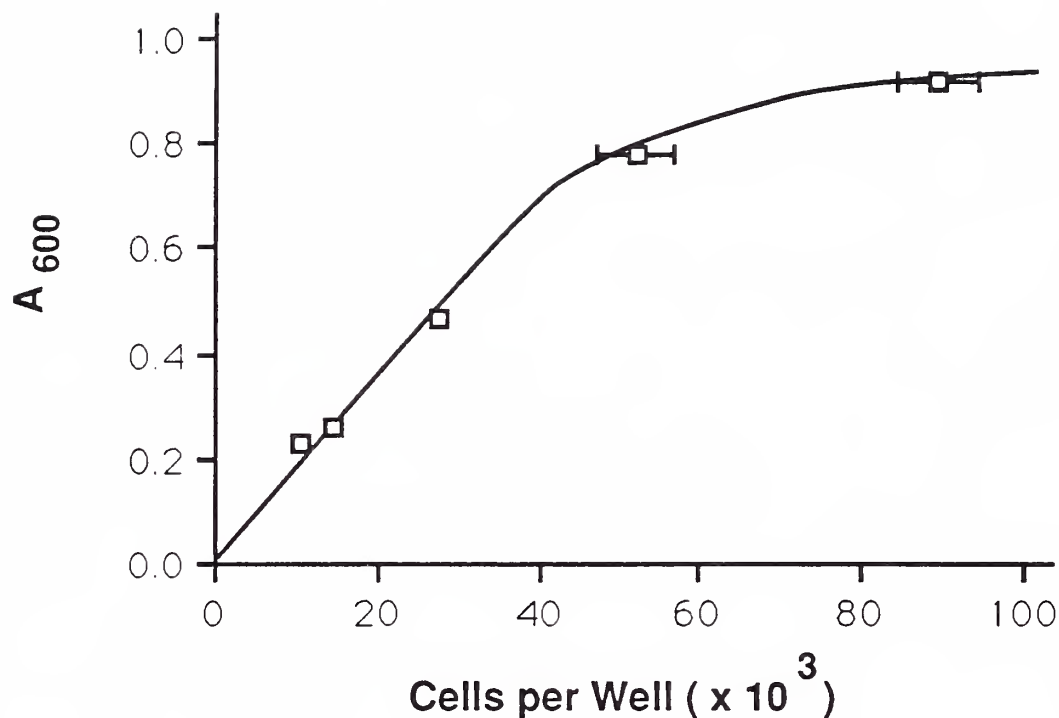
X	R	Name	Cell Growth Inhibition $IC_{50} \pm S.E. (\mu M)$	MDR Ratio $\pm 95\% C.I.$
—Cl	$=CH-CH_2-N(CH_3)_2$	<i>cis</i> - 762 (B) <i>trans</i> - 762 (A)	$30 \pm 4$ $40 \pm 6$	$1.1 \pm 0.2^d$ $1.3 \pm 0.1^c$
—Cl	$=CH-CH_2-CH_2-N(CH_3)_2$	<i>cis</i> - chlorprothixene <i>trans</i> - chlorprothixene	$20 \pm 2$ $13 \pm 2$	$2.0 \pm 0.6^a$ $7.0 \pm 0.9^a$
—Cl	$=CH-CH_2-CH_2-N(CH_2CH_3)_2$	<i>cis</i> - 768 (B) <i>trans</i> - 768 (A)	$17 \pm 3$ $18 \pm 3$	$4.0 \pm 0.3^a$ $7.2 \pm 0.6^a$
—CF <sub>3</sub>	$=CH-CH_2-CH_2-N(CH_3)_2$	<i>cis</i> - 796 (A) <i>trans</i> - 796 (B)	$28 \pm 4$ $14 \pm 3$	$1.8 \pm 0.2^b$ $2.8 \pm 0.3^a$
—Cl	$=CH-CH_2-CH_2-N(CH_2)_5$	<i>cis</i> - 753 (B) <i>trans</i> - 753 (A)	$20 \pm 3$ $40 \pm 5$	$6.7 \pm 1.8^a$ $15.0 \pm 2.5^a$
—Cl	$=CH-CH_2-CH_2-N(CH_2)_5H$	<i>racemic</i> 751	$8 \pm 2$	$1.8 \pm 0.3^b$
—Cl	$=CH-CH_2-N(CH_2)_5-N-CH_2CH_2OH$	<i>trans</i> - 7006	$13 \pm 3$	$1.6 \pm 0.4^b$
—Cl	$=CH-CH_2-CH_2-N(CH_2)_5-N-CH_2CH_2OH$	<i>cis</i> - clopenthixol <i>trans</i> - clopenthixol	$20 \pm 2$ $15 \pm 2$	$2.6 \pm 0.4^a$ $15.0 \pm 2.3^a$
—CF <sub>3</sub>	$=CH-CH_2-CH_2-N(CH_2)_5-N-CH_2CH_2OH$	<i>cis</i> - flupenthixol <i>trans</i> - flupenthixol	$24 \pm 4$ $25 \pm 4$	$4.8 \pm 0.6^a$ $15.2 \pm 1.9^a$
—Cl	$-CH_2-CH_2-CH_2-N(CH_2)_5-N-CH_2CH_2OH$	789	$22 \pm 5$	$9.7 \pm 2.7^a$

<sup>a</sup>  $P < 0.001$ <sup>b</sup>  $P < 0.05$ <sup>c</sup>  $P < 0.01$ <sup>d</sup> P - not significant

MCF-7/DOX cells were treated as described in Table 2-1.  $IC_{50}$  values  $\pm$  S.E. and MDR Ratios with 95% confidence intervals and P- values for comparison of  $IC_{50}$ 's for cell growth inhibition by doxorubicin in the absence versus presence of each modifier were also determined as described in Table 2-1. Each value represents the mean of two to three separate experiments; each experiment was done in quadruplicate.

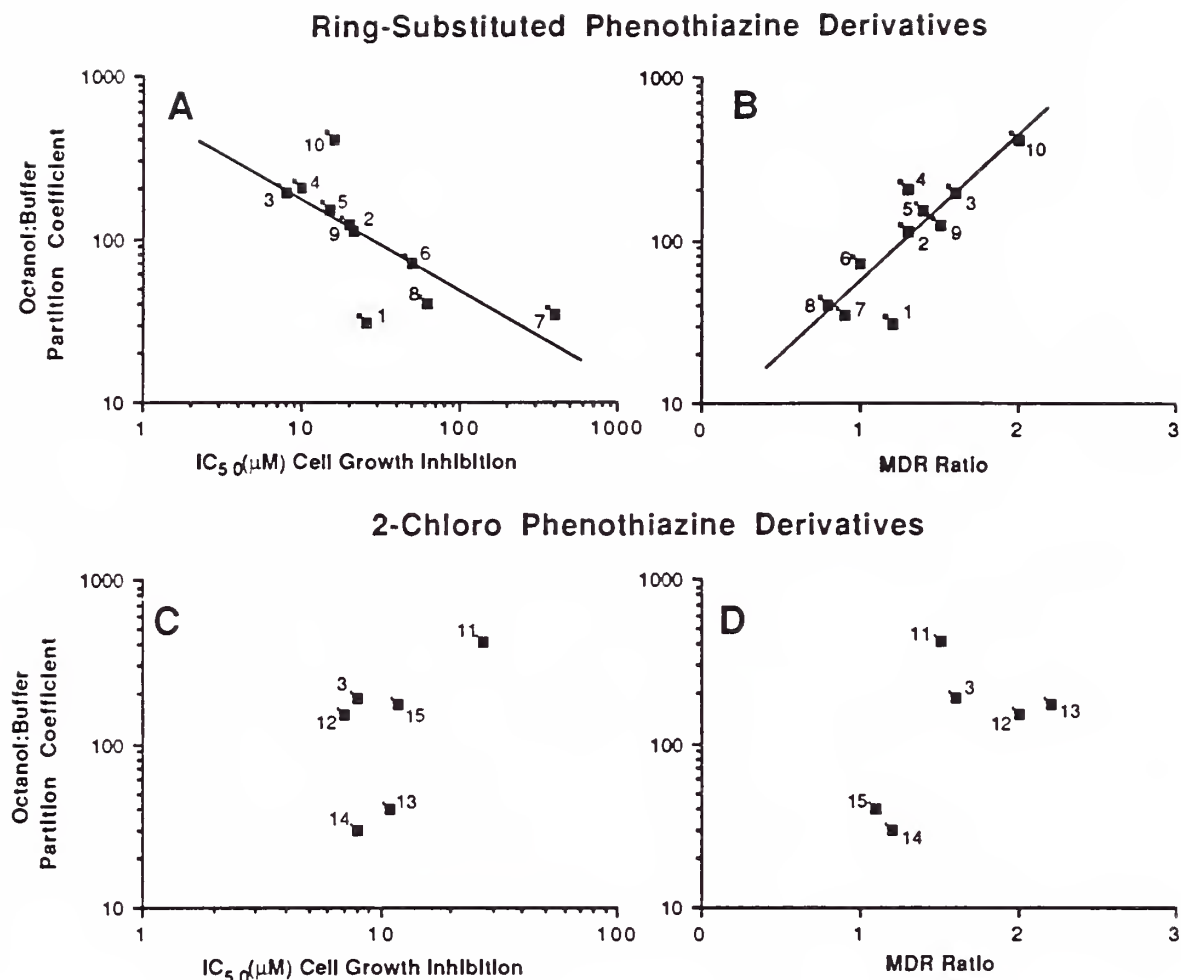






**Figure 2-1.** Correlation between absorbance ( $A_{600}$ ) of stained cellular protein and cell count. MCF-7/DOX cells were grown in 96-well microtiter plates at initial concentrations of 10,000 cells/well and enumerated after 24, 48, 72, 96 and 110 hour incubations by either absorbance spectrophotometry or with a Coulter Counter, as describe in Materials and Methods. Each point represents the mean of quadruplicate determiniations. Bars represent the standard error when greater than the symbol.



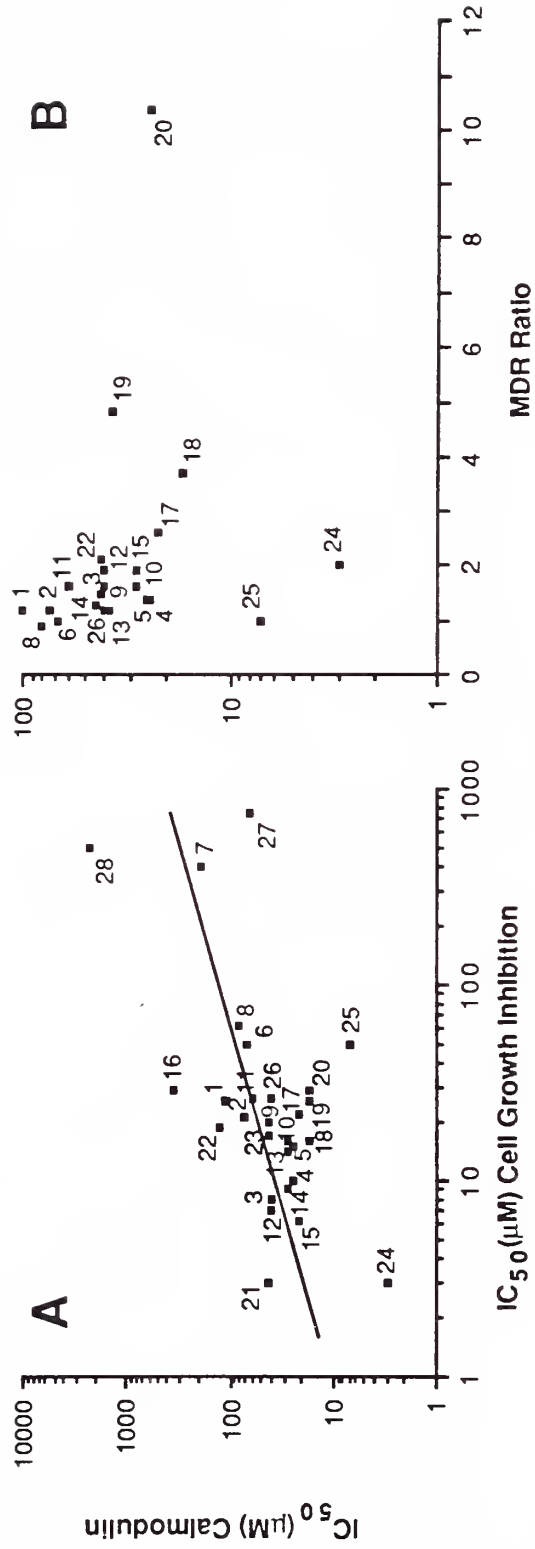


**Figure 2-2.** Relationship between hydrophobicity and activity of phenothiazine derivatives as antiproliferative and anti-MDR agents. (A) and (B): correlation between octanol:buffer partition coefficients, as previously determined (Prozialeck and Weiss, 1982), and the  $IC_{50}$ 's for inhibition of cell growth ( $r = -0.73$ ,  $P = 0.016$ ) and antagonism of MDR ( $r = 0.86$ ,  $P = 0.0015$ ) for a series of phenothiazine derivatives with ring-substitutions (Table 2-1). (C) and (D): lack of correlation between octanol:buffer partition coefficients and  $IC_{50}$ 's for inhibition of cell growth ( $r = 0.54$ ,  $P = 0.27$ ) and antagonism of MDR ( $r = 0.59$ ,  $P = 0.21$ ) for a series of 2-Cl substituted phenothiazine derivatives with side chain alterations (Tables 2-2 and 2-3). Numbered points represent; (1) promazine, (2) 1-chloropromazine, (3) chlorpromazine, (4) 3-chloropromazine, (5) 4-chloropromazine, (6) 7-hydroxychlorpromazine, (7) 3,8-dihydroxychlorpromazine, (8) 7,8-dihydroxychlorpromazine, (9) thiomethylpromazine, (10) trifluopromazine, (11) 2-chloro-10-[2-(dimethylamino)ethyl] phenothiazine, (12) 2-chloro-10-[4-(dimethylamino)butyl] phenothiazine, (13) didesmethylchlorpromazine, (14) desmethylchlorpromazine, and (15) chlorproethazine.



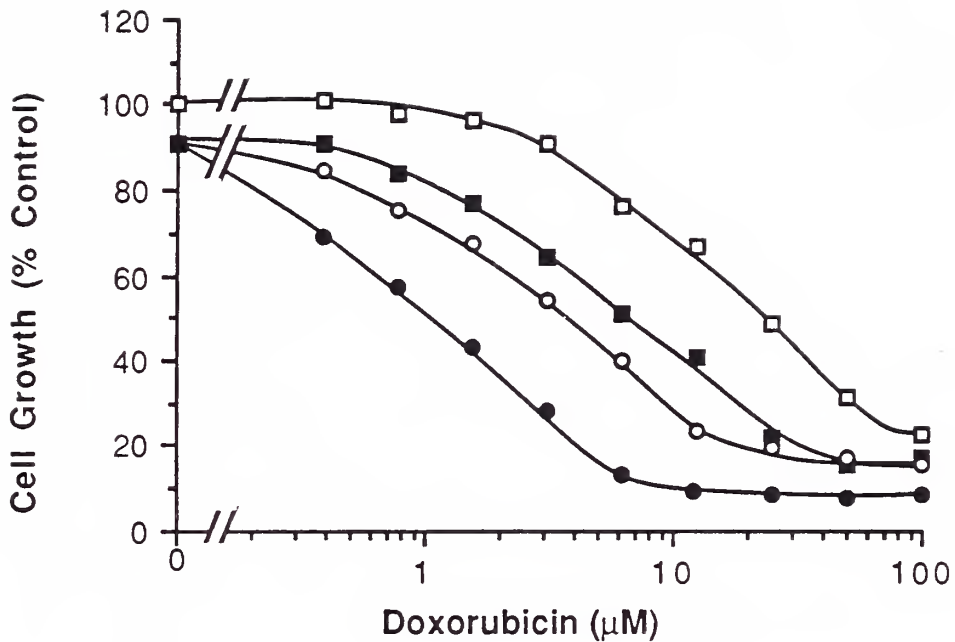


**Figure 2-3.** Relationship between anti-calmodulin activity and antiproliferative or anti-MDR activity for phenothiazine derivatives. (A): correlation between the  $IC_{50}$ 's for inhibition of calmodulin-induced activation of phosphodiesterase and the  $IC_{50}$ 's for inhibition of cell growth ( $r = 0.58$ ,  $P = 0.0009$ ) for phenothiazine derivatives. (B): lack of correlation between the  $IC_{50}$ 's for inhibition of calmodulin-induced activation of phosphodiesterase and antagonism of MDR by modifiers used at a standard concentration of  $3 \mu M$  ( $r = 0.17$ ,  $P = 0.44$ ) for phenothiazine derivatives. All  $IC_{50}$  values for the inhibition of calmodulin are from Prozialeck and Weiss (1982) and represent the concentration of drug necessary to inhibit by 50% the calmodulin-induced activation of the  $Ca^{++}$ -dependent form of cyclic nucleotide phosphodiesterase.  $IC_{50}$  values for the inhibition of cellular proliferation are from Tables 2-1 to 2-6. MDR Ratios are the mean of two or more experiments, each run in quadruplicate, and were determined as described in Materials and Methods, except that an equimolar concentration ( $3 \mu M$ ) of each modifier was used instead of an equitoxic concentration. Numbered points represent; (1) promazine, (2) 1-chloropromazine, (3) chlorpromazine, (4) 3-chloropromazine, (5) 4-chloropromazine, (6) 7-hydroxychlorpromazine, (7) 3,8-dihydroxychlorpromazine, (8) 7,8 dihydroxychlorpromazine, (9) thiomethylpromazine, (10) trifluopromazine, (11) 2-chloro-10-[2-(dimethylamino)ethyl] phenothiazine, (12) 2-chloro-10-[4-(dimethylamino)butyl] phenothiazine, (13) didesmethylchlorpromazine, (14) desmethylchlorpromazine, (15) chlorproethazine, (16) promethazine, (17) prochlorperazine, (18) trifluoperazine, (19) *cis*-flupenthixol, (20) *trans*-flupenthixol, (21) quinacrine, (22) imipramine, (23) 2-chloroimipramine, (24) penfluridol, (25) pimozide, (26) R-6033, (27) haloperidol, and (28) chlorpromazine sulfoxide.









**Figure 2-4.** Effect on the sensitivity of MDR cells to doxorubicin by PTZs and structurally related modifiers. MCF-7/DOX cells were exposed to 0-100  $\mu$ M doxorubicin for 48 hours in the absence (□) or presence of fluphenazine (■), cis-flupenthixol (○), or trans-flupenthixol (●) at concentrations that alone produced 10% inhibition of cell growth. Cell growth was determined by a microtiter assay as described in Materials and Methods. Each point represents the mean of quadruplicate determinations which differed by less than 5%.



### Chapter III

## ***IN VITRO* CHARACTERIZATION OF THE CHEMOSENSITIZING EFFECT OF THE THIOXANTHENES**

### **A. Introduction**

The analysis of structural determinants important for PTZs' anti-MDR activity, discussed in the previous chapter, enabled the identification of a novel class of chemosensitizing agents, the thioxanthenes. As outlined in Section I.B.2., the efficacy of chemosensitizers may be best evaluated by studying their effect on cytotoxic drug resistance, cross-resistance and drug accumulation in carefully defined MDR cell lines. The discovery that *trans*-thioxanthene isomers more effectively potentiate doxorubicin in MCF-7/DOX cells than *cis*-isomers provides a unique opportunity to probe the stereoisomeric specificity of these chemosensitizers on MDR, and to compare their anti-MDR activities with their degree of stereoisomerism in other cellular and enzymatic systems, as a means of better defining their possible mechanism(s) of action.

Accordingly, studies were performed comparing the effects the thioxanthene chemosensitizers *cis*- and *trans*-flupenthixol, the PTZ homolog fluphenazine, and the structurally unrelated chemosensitizer verapamil, on primary drug resistance, cross-resistance, and accumulation in several MDR cell lines, including three well-characterized, P-gp expressing human and mouse MDR lines (MCF-7/DOX, KB-V1, and P388/DOX), a murine fibroblast cell line transfected with a human *mdr1* gene expression vector and selected for P-gp overexpression and resistance to colchicine (3T3/MDR1), and an 'atypical' MDR cell line which displays characteristics of MDR, including cross-resistance and decreased drug accumulation, but does not overexpress P-gp. In addition, the thioxanthene isomers *cis*- and *trans*-flupenthixol were studied for their potency and stereospecificity as inhibitors of CaM and PKC, cellular enzymes potentially important for reversing MDR.



## B . Materials and Methods

### 1 . Cell Lines and Culture

MCF-7 and MCF-7/DOX human breast cancer cells were maintained as described in Section II.B.1. The latter cell line has been shown to uniquely overexpress P-gp and the glutathione-S-transferase  $\pi$  isozyme (12, 56).

Human epidermoid carcinoma KB-3-1 cells and the vinblastine selected MDR subline, KB-V1 (kindly supplied by Dr. Michael M. Gottesman, National Cancer Institute, Bethesda, MD), were grown in monolayer culture at 37° C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and L-glutamine, in the continuous presence of 1  $\mu$ g/ml vinblastine. These cells are  $\geq$  200-fold resistant to vinblastine, doxorubicin and colchicine (206), demonstrate decreased drug accumulation (64), overexpress P-gp (236), and possess a 100-fold amplification of the *mdr1* gene (188).

Murine leukemic P388 cells and the classic MDR cell line P388/DOX (from Dr. Ram Ganapathi, Cleveland Clinic Foundation, Cleveland, OH) were grown in suspension in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 10  $\mu$ M 2-mercaptoethanol.

Murine NIH 3T3 fibroblasts and a MDR 3T3 line (3T3/MDR1) transfected with a retrovirus expression vector containing a full-length cDNA from the human *mdr1* gene (235) and selected for resistance to colchicine (kindly provided by Drs. Michael M. Gottesman and Steven Currier) were grown in monolayer in RPMI 1640 supplemented with 10% fetal calf serum, in the continuous presence of 1  $\mu$ g/ml colchicine.

MCF-7/MITOX cells (generously supplied by Dr. William S. Dalton, University of Arizona Cancer Center, Tucson, AZ), selected for resistance to mitoxantrone, were grown similarly to the other MCF-7 lines, except that RPMI 1640 medium was supplemented with 10% fetal bovine serum, and cells were continuously grown in  $8 \times 10^{-8}$  M mitoxantrone. These cells display characteristics of MDR including cross-resistance to anthracyclines and *Vinca* alkaloids and decreased drug accumulation, but do not overexpress P-gp.

Cell lines grown in the presence of selecting agents were passaged 2 to 3 times out of



drug before use in experiments. Cell lines were routinely tested and found to be free of contamination by mycoplasma or fungi.

## 2. *In Vitro Drug Sensitivity Assays*

The individual antiproliferative effect of cytotoxic drugs, PTZs or thioxanthenes in the various cell lines were determined using microtiter spectrophotometric assays. All monolayer cell lines (MCF-7, KB, and NIH 3T3 derived lines) were assayed using the methylene blue cell staining method of Finlay *et al* (60) as described in Section II.B.2., with the following modifications. Correlations between cellular growth and spectrophotometric absorbance were performed for each cell line, as described for MCF-7/DOX cells in Figure 2-1, and the initial cell plating density and optimal assay conditions chosen to ensure optical density measurements fell on the linear portion of each curve.  $0.5 \times 10^4$  MCF-7 sensitive cells,  $1.0 \times 10^4$  MCF-7/DOX cells,  $1.2 \times 10^4$  MCF-7/MITOX cells,  $0.8 \times 10^4$  KB-3-1 cells,  $1.0 \times 10^4$  KB-V1 cells,  $0.6 \times 10^4$  NIH 3T3 cells, and  $1.0 \times 10^4$  3T3/MDR1 cells were dispensed in 100  $\mu$ l volumes medium into each well of 96-well microtiter plates. Cells were allowed to attach and grow for 24 hours, at which time 100  $\mu$ l of drug-containing medium was added. Cells were incubated at 37° C with drugs for 48 hours, except for MCF-7/MITOX cells, which required a 72 hour incubation for optimal growth, and stained with methylene blue, solubilized, and their optical density ( $A_{600}$ ) determined spectrophotometrically, as described in Section II.B.2.

Inhibition of growth for the P388 and P388/DOX suspension cell lines was determined using the modified MTT assay (30), based on the ability of live cells to reduce a tetrazolium based compound 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan product that can be measured spectrophotometrically. Briefly,  $0.5 \times 10^4$  P388 or P388/DOX cells in 100  $\mu$ l aliquots growth medium were plated into each well of 96-well microtiter plates, and allowed to equilibrate for 2 to 4 hours. Drugs dissolved in small amounts of sterile water or ethanol (final concentration  $\leq 0.1\%$ ) and diluted in medium





were added in 100  $\mu$ l volumes at appropriate concentrations, with each condition performed in quadruplicate. Control wells received 100  $\mu$ l medium with vehicle, alone. Cells were incubated at 37° C for 3 days, at which time 50  $\mu$ l MTT (Sigma) solution (2 mg/ml PBS) was added to each well for an additional 4 hour incubation. Plates were centrifuged at 450 x g for 5 minutes, and cellular supernatants gently aspirated, leaving approximately 25  $\mu$ l medium in each well. Insoluble formazan crystals were dissolved by adding 150  $\mu$ l 100% DMSO to each well, and plates thoroughly mixed on a mechanical plate shaker for 10 minutes. The optical density of each well was determined by absorbance spectrophotometry at a wavelength of 550 nm using a Titertek Multiscan MCC/340 plate reader. Correlations between cell number and spectrophotometric absorption were performed as described for the the methylene blue staining assay, and plating densities and assay conditions chosen to ensure linearity. Inhibition of cell growth was expressed as a percentage of absorbance of vehicle-treated control cultures.

The relative resistance and cross-resistance of each cell line to various drugs was determined by dividing the IC<sub>50</sub> concentration for resistant cell lines to each drug by the IC<sub>50</sub> for that drug in the respective parental, sensitive cell line.

The effect of chemosensitizers on drug resistance and cross-resistance was studied by exposing cells to a range of concentrations of cytotoxic drugs in the absence or presence of equimolar concentrations of chemosensitizers that alone produced  $\leq$  10% inhibition of growth. Except where otherwise indicated, 5  $\mu$ M concentrations of chemosensitizers were used for all experiments. Dose-response curves were corrected for the inhibition of cell growth caused by chemosensitizers alone, and MDR Ratios, as defined in Section II.B.2., were calculated for each drug plus chemosensitizer combination.

$$\text{MDR Ratio} = \text{IC}_{50} \text{ Drug alone} \div \text{IC}_{50} \text{ Drug} + \text{Chemosensitizer}$$

### 3. *Cellular Accumulation of Thioxanthenes*

Duplicate aliquots of  $3 \times 10^6$  MCF-7/DOX cells in a total volume of 2 ml were incubated at 37° C for 3 hours in the presence of 0 - 100  $\mu$ M of each drug. Cells were



washed three times in cold PBS and centrifuged at 100 x g for 10 minutes, resuspended in 2 ml of 0.3 N HCl in 50% ethanol, and sonicated for 10 pulses at 200 watt seconds with a Tekmar cell sonicator (Tekmar, Cincinnati, OH). Following centrifugation at 1000 x g for 30 minutes, the cell supernatant was removed and assayed for drug concentration with a Perkin-Elmer 512 spectrofluorometer (Norwalk, CT). Optimal excitation and emission wavelengths for both thioxanthene isomers were determined to be 320 nm and 400 nm, respectively. Cellular drug content (nmoles/ $10^6$  cells) was computed from standard curves prepared with known amounts of drug in 0.3 N HCl in 50% ethanol.

#### 4. Cellular Accumulation of Doxorubicin

Sensitive and MDR MCF-7 cells were each seeded in three 40 ml volumes of medium at a density of  $1.67 \times 10^6$  cells/ml. 100  $\mu$ l of concentrated stock drug solutions were added to obtain a final doxorubicin concentration of 10  $\mu$ M, in the absence or presence of either equitoxic ( $IC_{10}$ ) concentrations of *cis*- or *trans*-flupenthixol (3  $\mu$ M and 6  $\mu$ M, respectively), or equimolar (5  $\mu$ M) concentrations of all chemosensitizers. Cells were incubated at 37° C for 3 hours. At various times after the addition of drugs, three 1.5 ml aliquots of cellular suspension were removed from each cell solution, immediately centrifuged for 60 sec at 11,000 x g, and washed three times with cold PBS using an Eppendorf 5415 microcentrifuge. Control samples were removed at time zero, immediately before drugs were added. Cell pellets were extracted with 0.3 N HCl in 50% ethanol, sonicated and centrifuged as described above. Cell supernatants were removed and assayed fluorometrically for doxorubicin content using excitation and emission wavelengths of 470 nm and 585 nm, respectively, as previously described (69). Cellular content of doxorubicin (pmoles/ $10^6$  cells) was computed from standard curves prepared with known amounts of drug. The presence of chemosensitizers was shown not to effect the absorbance or emission spectra of doxorubicin.

#### 5. Isobologram Analyses



After determining the  $IC_{50}$  for doxorubicin and the  $IC_{50}$  for individual chemosensitizers against MCF-7/DOX cells, a series of dose-response curves to *trans*-flupenthixol in the presence of fixed concentrations of doxorubicin were done by the microtiter assay system. The individual concentrations of doxorubicin plus *trans*-flupenthixol that together resulted in 50% inhibition of growth of MCF-7/DOX cells were plotted and this  $IC_{50}$  isobole compared to the calculated line of additivity, using criteria previously described (22).

Similarly, a MDR isobologram analysis was designed to study the *in vitro* additivity, synergy or competitiveness of *trans*-flupenthixol plus verapamil for reversing MDR. Accordingly, multiple dose-response curves for MCF-7/DOX cells to doxorubicin were performed, in the absence or presence of various combinations of *trans*-flupenthixol plus verapamil. Those concentrations of chemosensitizers that together caused a 15-fold reversal of MCF-7/DOX cell resistance to doxorubicin were graphed and compared to a theoretical line of chemosensitizing additivity. An MDR Ratio = 15 was arbitrarily chosen as the defined 'response,' for which particular doses of chemosensitizers were necessary to achieve, analogous to the  $IC_{50}$  response as defined for a traditional isobologram.

## 6. Inhibition of Calmodulin by Thioxanthenes

The activity of bovine brain CaM (Calbiochem) was determined by its ability to activate a CaM-dependent cyclic nucleotide phosphodiesterase prepared from rat cerebrum, as previously described (137). Phosphodiesterase activity was measured by the luciferin-luciferase method (246). One unit of CaM was defined as the amount needed to produce 50% of the maximal activation of phosphodiesterase. Briefly, to assay CaM activation, each reaction vessel contained 50 mM glycylglycine buffer, pH 8.0, containing 25 mM ammonium acetate, 3 mM  $MgCl_2$ , 0.1 mM  $CaCl_2$ , 0.25 mM phosphoenolpyruvate, 5 mM dithiothreitol, 1 nM ATP, 1  $\mu$ g myokinase, 0.5  $\mu$ g pyruvate kinase, 0.4 mM cyclic AMP, and the phosphodiesterase preparation in a total volume of 100  $\mu$ l. Samples were incubated at 37° C for 30 min, and then placed in a boiling water bath for 5 min to destroy the



phosphodiesterase activity. After cooling, an additional 25  $\mu$ l of glycylglycine buffer containing 2  $\mu$ g of myokinase and 1  $\mu$ g pyruvate kinase were added and the sample reincubated for 30 min at 37° C. The firefly luciferin-luciferase reagent was added, and the hydrolysis of cyclic AMP measured using a Luminescence Biometer. The effect of thioxanthenes on the activity of calmodulin was determined by their ability to inhibit the activation of phosphodiesterase in the presence of 10 units of calmodulin. The specificity of this assay was shown by determining the basal inhibition of phosphodiesterase in the absence of CaM.

### ***7. Inhibition of Protein Kinase C by Thioxanthenes***

PKC was partially purified by modifications of previously described methods (57, 124). Briefly,  $1 \times 10^8$  MCF-7/DOX cells were washed once with PBS by centrifugation at 100 x g at 4° C and the pellet resuspended in 1.5 ml of 20 mM Tris HCl buffer containing 2 mM EDTA, 0.5 mM EGTA, 2 mM PMSF and 5 mM 2-mercaptoethanol, pH 7.5. The cells were allowed to swell for 5 minutes and then gently homogenized with 30 strokes of a Dounce Homogenizer with a loose fitting glass pestle. Sucrose was added to a final concentration of 0.33 M and the homogenate centrifuged at 100,000 x g for 1 hour. The soluble supernatant fraction was then added batchwise to DE52 preequilibrated with sample buffer. The resin was washed twice with the sample buffer containing 0.33 M sucrose and the enzyme was eluted with 20 mM Tris HCL buffer containing 100 mM NaCl, 2 mM PMSF and 5 mM 2-mercaptoethanol. All procedures were carried out at 4° C. The activity of the enzyme was purified approximately 15-fold.

The activity of PKC was determined by measuring the incorporation of  $^{32}$ P from [ $\gamma$ - $^{32}$ P]-ATP into lysine rich histone by modifications of previously described methods (169). A reaction mixture consisted of 40  $\mu$ g histone, 4  $\mu$ g/ml diolein, with or without 25  $\mu$ g/ml phosphatidylserine and 50  $\mu$ l of the partially purified enzyme (approximately 1  $\mu$ g) in 20 mM Tris HCl containing 10 mM  $\text{MgCl}_2$ , 0.5 mM  $\text{CaCl}_2$ , pH 7.5. The reaction was initiated by the addition of 20  $\mu$ M ATP containing 0.66 pmol of [ $\gamma$ - $^{32}$ P]-ATP ( $6.66 \times 10^6$





cpm/pmol) in a final volume of 200  $\mu$ l. Following a 10 minute incubation at 30° C, 25  $\mu$ l aliquots of the reaction mixture were spotted onto P81 cation exchange paper. The filters were washed 3 times in 75 mM phosphoric acid and counted in 5 ml of Ecoscint using a Beckman LS 7500 liquid scintillation counter. Assay conditions were chosen to insure linearity of the reaction with time of incubation and concentration of tissue.

## 8. *Drugs and Reagents*

Fluphenazine was provided by Dr. S. J. Lucania (E. R. Squibb and Sons), *cis*- and *trans*-flupenthixol by Dr. John Hyttel (H. Lundbeck, Copenhagen, Denmark), bleomycin (Blenoxane) by Dr. Emmanuel Losada (Bristol Laboratories, Wallingford, CT), and mitoxantrone by Hank Durivage (Section of Medical Oncology, Yale University School of Medicine). Doxorubicin, vinblastine sulfate, colchicine, verapamil, adenosine monophosphate, L-phosphatidyl-L-serine, 1,3-diolein, and histone (type III-S) were obtained from Sigma. Myokinase and pyruvate kinase were obtained from Boehringer-Mannheim (Indianapolis, IN) and firefly luciferin-luciferase was from E.I. Dupont de Nemours & Co. (Wilmington, DL). [ $\gamma$ - $^{32}$ P]-ATP was obtained from New England Nuclear Research Products, Inc. (Boston, MA). Whatman DE52 ion exchange cellulose was purchased from Macalaster Bicknell (New Haven, CT). P81 cellulose phosphate paper was purchased from Whatman (Hillsboro, OR). Other reagents were of analytical grade and were obtained from general commercial sources.

## C. Results

### 1. *Inhibition of Cellular Proliferation by Chemosensitizers*

Table 3-1 shows the IC<sub>50</sub> concentrations for inhibition of cell growth by *cis*-flupenthixol, *trans*-flupenthixol, fluphenazine or verapamil in each of the cell lines studied. In general, the P388 sensitive and P388/DOX cells were more sensitive, and the NIH 3T3 and 3T3/MDR1 cells more resistant to the antiproliferative effects of each of the four chemosensitizers used alone, than the MCF-7 or KB derived cell lines. *Cis*-flupenthixol,



*trans*-flupenthixol and fluphenazine were 2- to 10-fold more potent antiproliferative agents than verapamil, but were equitoxic to each other in each of the cell lines used. In addition, the PTZ and thioxanthene chemosensitizers were equally or slightly more toxic to each of the sensitive cell lines when compared to their MDR sublines (1- to 3-fold); no MDR line was collaterally sensitive to any of the chemosensitizers. The concentration of chemosensitizers necessary to inhibit growth by less than 10% was also determined for each cell line (data not shown), and these IC<sub>10</sub> doses used for experiments combining chemosensitizers with cytotoxic drugs. 5  $\mu$ M concentrations were found to be appropriate for all four chemosensitizers in each cell line except the P388 lines, in which 1  $\mu$ M PTZ and thioxanthenes were used, and the NIH 3T3 lines, in which up to 12  $\mu$ M chemosensitizer could be used without significant toxicity.

## ***2. Relative Resistance and Cross-Resistance of Cell Lines to Cytotoxic Drugs***

Table 3-2 shows the IC<sub>50</sub> values for the inhibition of cell growth by several different cytotoxic drugs in each of the cell lines, and the calculated degree of resistance (relative resistance) displayed to each drug. As can be seen, each of the resistant sublines displayed a typical MDR phenotype, with significant cross-resistance to anthracyclines (doxorubicin, mitoxantrone), *Vinca* alkaloids (vinblastine), and other natural product drugs (colchicine), but little or no resistance to bleomycin. Most, but not all, of the MDR cell lines were more resistant to the drug used for selection (primary resistance), than to the other drugs tested (cross-resistance). For example, MCF-7/MITOX cells were 100-fold resistant to mitoxantrone, but only 50-fold, 20-fold and 10-fold resistant to doxorubicin, colchicine, and bleomycin, respectively. Conversely, MCF-7/DOX cells were 200-fold resistant to the selecting agent doxorubicin, but displayed greater cross-resistance (400-fold) to colchicine. The MDR KB-V1, P388/DOX and 3T3/MDR1 lines all possessed greater levels of primary than cross-resistance.



### 3. Reversal of Multidrug Resistance by Chemosensitizers

To compare the effects of each of the chemosensitizers for reversing primary and cross-resistance to cytotoxic drugs in sensitive and MDR cells, the  $IC_{50}$ 's for cells to each drug in the absence and presence of nontoxic, equimolar concentrations were determined, and MDR Ratios calculated for each. Table 3-3 shows the fold reversal of resistance (MDR Ratio) of each cell line to different drugs caused by *cis*-flupenthixol, *trans*-flupenthixol, fluphenazine and verapamil. None of the chemosensitizers displayed significant activity in potentiating the effect of any of the cytotoxic drugs tested in the sensitive cell lines, MCF-7, KB-3-1, P388 or NIH 3T3, except for verapamil plus doxorubicin in P388 cells (MDR Ratio = 2.3), and fluphenazine and vinblastine in MCF-7 cells (MDR Ratio = 2.0). Similarly, the chemosensitizers did not alter the primary resistance to mitoxantrone or cross-resistance to doxorubicin and vinblastine in the non-P-gp expressing MDR cell line MCF-7/MITOX, with 5  $\mu$ M of each resulting in MDR Ratios = 1.0.

All four chemosensitizers were effective in partially reversing primary or cross-resistance in the remaining, well-characterized MDR cell lines, with *trans*-flupenthixol possessing the greatest activity. For example, in the MCF-7/DOX, KB-V1 and P388/DOX MDR cells, *trans*-flupenthixol caused an 8- to 36-fold, 20- to 40-fold, and 4- to 7-fold reversal, respectively, of cell resistance to doxorubicin, vinblastine and colchicine. *Trans*-flupenthixol was stereospecific in its anti-MDR effects, since in these three MDR cell lines, equimolar concentrations of *trans*-flupenthixol caused 1.5- to 2-fold greater antagonism of MDR than its stereoisomer, *cis*-flupenthixol, and 2- to 12-fold greater antagonism than its PTZ homolog fluphenazine. In addition, *trans*-flupenthixol was up to 3-fold more effective for reversing MDR than the chemosensitizer verapamil. Conversely, in the colchicine selected 3T3/MDR1 cell line, *trans*-flupenthixol was a 1- to 2-fold less effective chemosensitizer than its isomer *cis*-flupenthixol, or verapamil, though still significantly more effective than fluphenazine. However, both thioxanthenes and verapamil were extremely effective at reversing MDR in this *mdr1* transfected line, with nontoxic



concentrations of 12  $\mu\text{M}$  causing approximately 40-fold reversal of the 100-fold colchicine resistance, and fully reversing the 20-fold doxorubicin cross-resistance to doxorubicin. Finally, the thioxanthenes and verapamil caused a greater reversal of resistance to doxorubicin or vinblastine than colchicine in those cells selected with either of the former drugs (MCF-7/DOX and KB-V1), while the chemosensitizers caused a significantly greater potentiation of colchicine than doxorubicin in the 3T3/MDR1 colchicine selected cells.

#### 4. Drug Accumulation Studies

It was next determined whether the difference in the anti-MDR activity of the thioxanthenes could be attributed to differences in their cellular accumulation. After a 3 hour incubation in 3 - 100  $\mu\text{M}$  concentrations of thioxanthenes, cell associated *cis*- and *trans*-flupenthixol concentrations (nmoles/ $10^6$  cells) were  $1.29 \pm 0.11$  versus  $0.41 \pm 0.04$  at 3  $\mu\text{M}$  ( $P < 0.001$ ),  $4.34 \pm 0.65$  versus  $2.22 \pm 0.16$  at 10  $\mu\text{M}$  ( $P < 0.001$ ), and  $14.52 \pm 0.66$  versus  $12.2 \pm 330$  at 0.66  $\mu\text{M}$  ( $P > 0.1$ ).

The effect of both equitoxic and equimolar concentrations of chemosensitizers on the accumulation of doxorubicin in both sensitive and MDR cell lines was also studied. Figure 3-1 demonstrates that after a 3 hour incubation in 10  $\mu\text{M}$  doxorubicin, by which time steady-state concentrations were reached, MCF-7/DOX cells accumulated approximately 10-fold less doxorubicin than the sensitive cell line. The addition of 3  $\mu\text{M}$  *cis*- or 6  $\mu\text{M}$  *trans*-flupenthixol ( $\text{IC}_{10}$  concentrations) had no significant effect on the accumulation of doxorubicin in the sensitive MCF-7 line. However, they increased by 2.4 and 4.6-fold, respectively, the accumulation of doxorubicin in the resistant MCF-7/DOX cells.

Similarly, Figure 3-2 shows that after a 3 hour incubation with equimolar (5  $\mu\text{M}$ ) concentrations of *cis*-flupenthixol, *trans*-flupenthixol or verapamil, *trans*-flupenthixol caused a 5-fold increase in doxorubicin accumulation, while *cis*-flupenthixol caused a 3-fold and verapamil caused a 4-fold increase, in MCF-7/DOX cells.





## 5. *Isobologram Analyses*

To rigorously study the magnitude of potentiation of doxorubicin by *trans*-flupenthixol, their multiple drug effects were studied by isobologram analysis. Figure 3-3 demonstrates the synergistic action of doxorubicin and *trans*-flupenthixol, evident by comparing the actual concentrations necessary for 50% inhibition of cell growth to those predicted for drugs which are simply additive.

To determine whether *trans*-flupenthixol and verapamil are additive, competitive, or synergistic in their ability to potentiate the cytotoxicity of doxorubicin in MCF-7/DOX cells, a series of dose-response curves to doxorubicin were performed in the presence of various combinations of the two chemosensitizers. Figure 3-4 shows a plot of the concentrations that either alone or in combination caused a 15-fold reversal of doxorubicin resistance in these cells. As can be seen, *trans*-flupenthixol and verapamil were exactly additive in their chemosensitizing effects.

## 6. *Potency of Chemosensitizers*

To compare the anti-MDR potency of *trans*-flupenthixol versus verapamil, dose-response curves to doxorubicin in the absence and presence of various concentrations of individual chemosensitizers were performed. Figure 3-5 shows that while both chemosensitizers displayed linearly increasing anti-MDR effects with increasing doses, *trans*-flupenthixol was 2- to 3-fold more potent than verapamil in producing equivalent effects. For example, to cause a 15-fold reversal of MDR (MDR Ratio = 15), 5  $\mu$ M *trans*-flupenthixol versus 12  $\mu$ M verapamil were necessary.

## 7. *Effect of Thioxanthenes on Calmodulin and Protein Kinase C*

Since PTZs and thioxanthenes are both known to antagonize CaM, the effect of *cis*-flupenthixol, *trans*-flupenthixol and fluphenazine as inhibitors of a CaM-dependent form of cyclic nucleotide phosphodiesterase were determined and compared to their chemosensitizing effects. Table 3-4 shows that *cis*-flupenthixol, *trans*-flupenthixol and



chemosensitizing effects. Table 3-4 shows that *cis*-flupenthixol, *trans*-flupenthixol and fluphenazine were all potent CaM antagonists. However, their anti-CaM activity did not correlate with their effects on MDR. Specifically, while *trans*-flupenthixol was a slightly more potent CaM antagonist than *cis*-flupenthixol ( $IC_{50} = 7 \mu M$  versus  $11 \mu M$ ), both were slightly less potent than fluphenazine ( $IC_{50} = 5 \mu M$ ), a far less effective chemosensitizer than either thioxanthene.

*Cis*- and *trans*-flupenthixol were also examined for their effect on the inhibition of PKC. Table 3-4 shows that the thioxanthenes were extremely similar in their anti-PKC effect, with the *trans*-isomer being slightly more potent (1.4-fold).

#### D. Discussion

The results presented in this chapter characterize the stereospecific effects of *trans*-flupenthixol and several related chemosensitizers on MDR, and suggest that the thioxanthene class of MDR antagonist mediates this effect through P-gp. Specifically, it was found that the chemosensitizing activity of the thioxanthenes, verapamil, and to a lesser extent fluphenazine, were limited to 'classic' MDR cell lines. For example, *trans*-flupenthixol caused significant reversal of primary and cross-resistance in MCF-7/DOX, KB-V1, P388/DOX, and 3T3/MDR1 cell lines, each known to display decreased drug accumulation and an increased expression of P-gp or *mdr1* mRNA (56, 64, 71, 206, 235). However, neither *trans*-flupenthixol, nor the other chemosensitizers tested, caused any potentiation of drug toxicity in the MCF-7/MITOX multiple drug resistant cell line. It is interesting to note that while these cells display decreased drug accumulation and cross-resistance to a typical MDR spectrum of drugs, extensive studies utilizing Western blot analysis with several different monoclonal antibodies to P-gp and Northern blotting with cDNA probes to the *mdr1* gene have failed to detect any expression of P-gp mRNA or protein.<sup>1</sup> The mechanism of drug resistance is as yet unclear for these cells, although an increased amount of a 150 kDa membrane protein has been identified by gel electrophoresis

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<sup>1</sup>Dalton, W., personal communication.



The chemosensitizers studied did not significantly modulate the toxicity of drugs nor cause an increased accumulation of doxorubicin in sensitive, parental cell lines. These results together suggest that the chemosensitizing effects of the thioxanthenes and verapamil were mediated through a target(s) overexpressed in MDR cells, such as the putative drug efflux pump, P-gp (38). As discussed in Chapter I, a current hypothesis regarding the mechanism by which MDR cells reduce cellular accumulation of anthracyclines and other drugs is through the increased expression of this plasma membrane glycoprotein in MDR cells (27), and that compounds which antagonize MDR compete with cytotoxic drugs for specific drug-binding sites on the protein (44). Though calcium channel blockers such as verapamil can inhibit binding of a photoaffinity labelled vinblastine analog to P-glycoprotein, PTZs were far less effective (2). The failure of PTZs to block vinblastine binding to P-glycoprotein does not rule out the interaction of chemosensitizers with other sites on the protein. For example, Hamada and Tsuruo have recently demonstrated ATPase activity of the molecule (93), and shown that agents which inhibit active drug efflux, such as trifluoperazine and verapamil, cause an increase in P-glycoprotein ATPase activity, while doxorubicin and vincristine, agents which may interact with the putative drug-binding region, do not affect this ATPase activity (92). Alternatively, Center (32) demonstrated that trifluoperazine increased phosphorylation of this protein in MDR Chinese hamster lung cells and enhanced doxorubicin accumulation and cytotoxicity, suggesting the PTZs may indirectly affect P-gp.

However, the remarkably similar effects of *trans*-flupenthixol and verapamil in each of the sensitive and MDR cell lines strongly suggests that the thioxanthenes may share a common target with verapamil for reversing MDR. In support of this, it was shown in Figure 3-4 that *trans*-flupenthixol and verapamil were exactly additive in their *in vitro* anti-MDR effect in MCF-7/DOX cells, implicating a common pharmacologic mechanism. Therefore, the decreased P-gp binding shown for PTZs (2) may actually explain the relative lack of potency and low MDR Ratios found for fluphenazine, trifluoperazine, and the many other PTZs tested for chemosensitizing activity in Chapter II, rather than implicating an



alternative mechanisms of reversal.

It will be critical to the understanding of the thioxanthenes' mechanism of action and stereospecificity in antagonizing MDR, to study their interaction with P-gp. In fact, efforts are currently in progress to determine the ability of *trans*-flupenthixol, *cis*-flupenthixol and fluphenazine to competitively inhibit the binding of photoactivatable vinblastine analogs to KB-V1 membrane P-gp, similar to previous studies with verapamil (44). Also, radiolabelled [ $^3\text{H}$ ]-*trans*-flupenthixol has recently been synthesized, and studies measuring its specificity and saturability binding to MDR KB-V1 membrane vesicle preparations will commence shortly.

It is extremely interesting that while *trans*-flupenthixol was a more potent and effective chemosensitizer than its stereoisomer, *cis*-flupenthixol, in each of the *in vitro* (MCF-7/DOX, KB-V1) or *in vivo* (P388/DOX) derived MDR cell lines, *trans*-flupenthixol was equal, or even less effective than *cis*-flupenthixol at antagonizing MDR in the 3T3/MDR1 line (Table 3-3). The fact that this particular fibroblast line transfected with an *mdr1* gene expression vector should theoretically display a particularly 'pure' form of P-gp mediated MDR suggests several possible explanations. First, Roninson's group has elegantly shown that mutations in the *mdr1* gene caused by colchicine selection result in a single amino acid change at position 185 of the transcribed P-gp protein, and that this genetic event is associated with a preferential increase in resistance to colchicine, possibly due to alterations in the affinity of P-gp drug binding sites (40). Since the 3T3/MDR1 transfected cell line used in the present studies was also selected with colchicine, in contrast to the other MDR cell lines used (selected with doxorubicin or vinblastine), it is intriguing to speculate that the apparent loss or reversal of flupenthixol's stereospecificity for antagonism of MDR may also be due to this mutation, thereby altering the affinity of a chemosensitizer and/or drug binding domain for *cis*- versus *trans*-flupenthixol. This may also explain why all the chemosensitizers caused a greater reversal of colchicine resistance than doxorubicin or vinblastine resistance in this cell line alone. Indeed, if this hypothesis is true, it may be





possible to target individual MDR subtypes, defined by their particular pattern of cross-resistance and *mdr1* sequence, with chemosensitizers that preferentially bind to that P-gp molecule.

Alternatively, *cis*- and *trans*-flupenthixol may possess equivalent effects on P-gp in all MDR cell lines, but have stereospecific effects on additional cellular targets which regulate or modulate MDR in certain cell lines. This hypothesis could explain *trans*-flupenthixol's loss of stereospecificity in the genetically manipulated *mdr1* transfectant, a line which may lack regulatory mechanisms or important co-induced gene products common to the other MDR cell lines. To begin exploring this possibility, the effect of thioxanthenes and PTZs on several cellular enzymes which may have a regulatory role in MDR was examined.

Similar to previous reports (161), *cis*- and *trans*-flupenthixol were found to be nearly identical antagonists of CaM, and were less potent than their PTZ homolog, fluphenazine (Figure 3-6). This data supports that presented in Chapter II demonstrating the lack of correlation between antagonism of CaM and reversal of MDR for PTZs.

Several groups have suggested that PKC may play an important role in MDR (58, 105). Drugs that stimulate PKC, such as the phorbol esters, produce increased levels of anthracycline resistance in MCF-7/DOX cells, and induce a MDR-like phenotype in sensitive MCF-7 cells (59). These effects were reversed by PTZs at concentrations similar to those used in the present study. Furthermore, MCF-7/DOX cells, in particular, have up to a 15-fold increased level of PKC activity compared to the parental MCF-7 cells (59, 166). In isolated systems, the concentrations of PTZs required to inhibit PKC have been reported to be many fold higher than those presently found sufficient to antagonize MDR. For example, in one study, the  $IC_{50}$ 's for inhibition of PKC by trifluoperazine, chlorpromazine and fluphenazine were 38 - 50  $\mu$ M, 59 - 84  $\mu$ M, and 78 - 114  $\mu$ M, respectively (200), 10- to 50-fold greater than those found to antagonize MDR. Many thioxanthenes are particularly poor inhibitors of PKC, with  $IC_{50}$ 's in the millimolar range reported (200), though the PKC inhibitory activity of *cis*- or *trans*-flupenthixol have not been previously studied. Figure 3-7 demonstrates that both flupenthixol isomers are potent inhibitors of PKC purified from



3-7 demonstrates that both flupenthixol isomers are potent inhibitors of PKC purified from MCF-7/DOX cells. While *trans*-flupenthixol is slightly more potent for this effect, it seems unlikely that the small difference (1.4-fold) can adequately explain their differences in chemosensitizing activity in this cell line (3-fold). Thus, while the activation and inhibition of PKC offers an attractive hypothesis for the modulation of MDR, it appears that the anti-MDR effects of the PTZs and thioxanthenes are not likely to be mediated solely through this enzyme.

Another cellular enzyme of particular relevance to the mechanism of MDR is glutathione-*S*-transferase, shown to be elevated in MCF-7/DOX cells (12). Recent evidence has shown that selectively reducing glutathione levels in MCF-7/DOX cells with buthionine sulfoximine leads to dramatically increased cellular verapamil toxicity, though causes no change in the sensitivity of cells to *trans*-flupenthixol.<sup>2</sup> Therefore, it appears that GST or the GSH redox system may be a target mediating verapamil, but not *trans*-flupenthixol, toxicity and/or chemosensitizing effect in MDR cells. Supporting this is preliminary data showing that *trans*-flupenthixol does not sensitize MCF-7 cells transfected with a GST $\pi$  expression vector to doxorubicin.<sup>3</sup>

Finally, a simple explanation for the differences in anti-MDR activity observed for the thioxanthene stereoisomers would be differences in their cellular accumulation and therefore differences in access to intracellular targets. However, MDR cells actually accumulate significantly more *cis*- than *trans*-flupenthixol at effective anti-MDR doses (3 - 10  $\mu$ M), the opposite of what would be expected if their potency as antagonists of MDR reflected differences in their intracellular accumulation. This implies that the difference in anti-MDR activity between these stereoisomeric thioxanthenes is indeed due to differences in their ability to interact with a unique cellular target(s). Since the most potent anti-MDR agent studied, *trans*-flupenthixol, reduced the 10-fold difference in doxorubicin accumulation

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<sup>2</sup>Ford, J.M. and W.N. Hait, manuscript in preparation.

<sup>3</sup>Ford, J.M., J.A. Moscow, K.H. Cowan, and W.N. Hait, manuscript in preparation.



chemotherapeutic drug efflux. However, because of the many cellular effects of the PTZs and thioxanthenes, it is possible these agents exert their effect through more than one mechanism.

In summary, *trans*-flupenthixol was a potent and effective chemosensitizer in each of the MDR cell lines studied, and appeared to function through a direct or indirect modulation or inhibition of P-gp function. *Trans*-flupenthixol's 2- to 3-fold greater potency and 1- to 3-fold greater efficacy than verapamil (Figure 3-5), along with its previously discussed lack of known clinical activity or side effects (Section II.D), indicate that future studies of the *in vivo* toxicity and efficacy of *trans*-flupenthixol in combination with doxorubicin and other chemotherapeutic agents are warranted to elucidate the potential of this drug for clinical use against MDR tumors.



TABLE 3-1

## Inhibition of Cellular Proliferation by Chemosensitizers Alone

Cell Line	IC <sub>50</sub> (μM) Chemosensitizers			
	<i>cis</i> -FPT	<i>trans</i> -FPT	FLU	VRP
MCF-7	8	9	18	100
MCF-7/DOX	24	25	23	200
MCF-7/MITOX	18	18	20	200
KB-3-1	15	15	15	35
KB-V1	32	17	32	35
P388	1.4	0.8	0.8	12
P388/DOX	0.9	0.6	1.2	12
NIH 3T3	30	30	60	70
3T3/MDR1	40	20	60	40

Inhibition of cell growth was determined by exposing cells to 0 - 100 μM *cis*-flupenthixol (*cis*-FPT), *trans*-flupenthixol (*trans*-FPT), flupenazine (FLU) or verapamil (VER) for 48 to 72 hours. IC<sub>50</sub> is the mean concentration that produced 50% inhibition of cell growth compared to vehicle-treated controls, as described in Materials and Methods. All values represent the mean of two to three separate experiments; each experiment was done in quadruplicate.





**TABLE 3-2**  
**Relative Resistance of Cells to Cytotoxic Drugs**

Cell Line	IC <sub>50</sub> (μM) and Relative Resistance				
	DOX	VLB	COLCH	MITO	BLEO
MCF-7	.06 (1)	.001 (1)	.005 (1)	1.0 (1)	15 (1)
MCF-7/DOX	12 (200)	0.1 (100)	2 (400)	20 (20)	15 (1)
MCF-7/MITOX	3 (50)	.03 (30)	0.1 (20)	100 (100)	150 (10)
KB-3-1	0.3 (1)	.002 (1)	.018 (1)	0.2 (1)	100 (1)
KB-V1	60 (200)	1 (500)	3 (166)	4 (20)	100 (1)
P388	.01 (1)	.002 (1)		.0013 (1)	1 (1)
P388/DOX	1 (100)	.04 (20)		.016 (12)	1 (1)
NIH 3T3	0.5 (1)		.05 (1)		
3T3/MDR1	10 (20)		5 (100)		

Inhibition of cell growth was determined by exposing cells to various concentrations of doxorubicin (DOX), vinblastine (VLB), colchicine (COLCH), mitoxantrone (MITO) or bleomycin (BLEO) for 48 to 72 hours. IC<sub>50</sub> is the mean concentration that produced 50% inhibition of cell growth compared to vehicle-treated controls, as described in Materials and Methods. The relative resistance of each cell line to different drugs was calculated by dividing the IC<sub>50</sub> of a MDR cell line to a drug by the IC<sub>50</sub> of its parental drug-sensitive cell line to the same drug. All values represent the mean of two to three separate experiments; each experiment was done in quadruplicate.



**TABLE 3-3**  
**Reversal of Multidrug Resistance by Chemosensitizers**

Cell Line	Drug	[CS] ( $\mu$ M)	MDR Ratio			
			<i>cis</i> -FPT	<i>trans</i> -FPT	FLU	VRP
MCF-7	DOX	5	1.0	1.0	1.0	1.0
	VLB	5	1.1	1.6	1.3	2.0
	MITO	5	1.5	1.7	1.0	1.0
	COLCH	5	1.1	1.0	1.0	1.0
MCF-7/DOX	DOX	5	7	15	13	3
	VLB	5	25	36	33	20
	COLCH	5	5	8	4	1.3
MCF-7/MITOX	MITO	5	1.0	1.0	1.0	1.0
	DOX	5	1.0	1.0	1.0	1.0
	VLB	5	1.0	1.0	1.0	1.0
KB-3-1	VLB	5	1.1	1.2	1.7	1.4
	DOX	5	1.0	1.0	1.0	1.0
	COLCH	5	1.1	1.0	1.1	1.2
KB-V1	VLB	5	20	40	15	10
	DOX	5	20	35	30	3
	COLCH	5	17	20	20	4
P388	DOX	1	1.4	1.0	2.3	1.7
	VLB	1	1.0	1.0	1.0	1.0
P388/DOX	DOX	1	5	7	6	2
	VLB	1	3	4	ND	3
NIH 3T3	COLCH	5	1.2	1.0	1.0	1.0
	DOX	5	1.0	1.0	1.0	1.0
3T3/MDR1	COLCH	5	10	10	20	3
	COLCH	12	40	37	43	10
	DOX	5	13	14	15	5
	DOX	12	36	20	40	13

Cells were exposed to various concentrations of doxorubicin (DOX), vinblastine (VLB), colchicine (COLCH) or mitoxantrone (MITO), in the absence or presence of *cis*-flupenthixol (*cis*-FPT), *trans*-flupenthixol (*trans*-FPT), flupenazine (FLU) or verapamil (VRP) at concentrations that alone produced  $\leq 10\%$  inhibition of cell growth ([CS]). Each MDR Ratio is the  $IC_{50}$  for cytotoxic drug alone divided by the  $IC_{50}$  for drug in the presence of chemosensitizer, and was derived as described in Materials and Methods. All values represent the mean of 2 to 3 separate experiments; each experiment was done in quadruplicate.



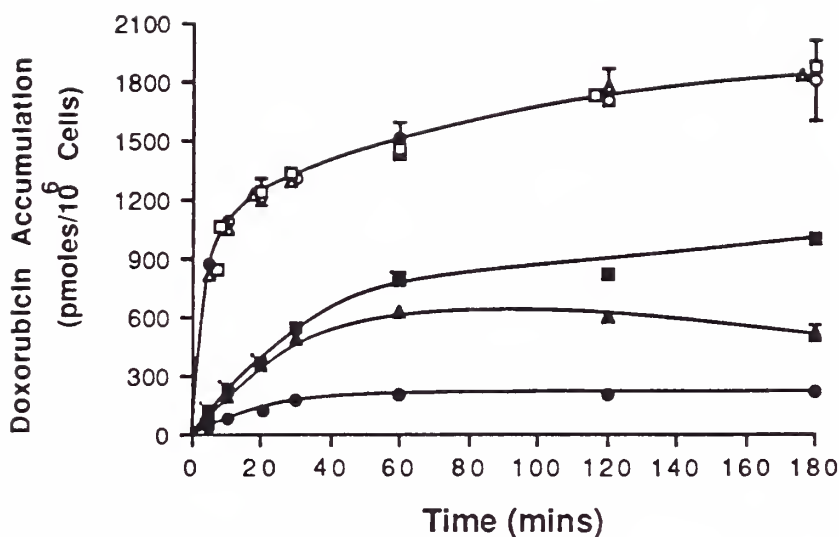
TABLE 3-4

**Inhibition of Calmodulin and Protein Kinase C by Chemosensitizers**

Chemosensitizer	IC <sub>50</sub> (μM) Chemosensitizers	
	CaM	PKC
<i>cis</i> -Flupenthixol	11	38
<i>trans</i> -Flupenthixol	7	28
Fluphenazine	5	

Antagonism of CaM by chemosensitizers was determined by their ability to inhibit by 50% the activation of a CaM-dependent form of cyclic nucleotide phosphodiesterase, as described in Materials and Methods. Effect of thioxanthenes on PKC was determined by their ability to inhibit by 50% the activity of enzyme purified from MCF-7/DOX cells approximately 15-fold, as described in Materials and Methods.

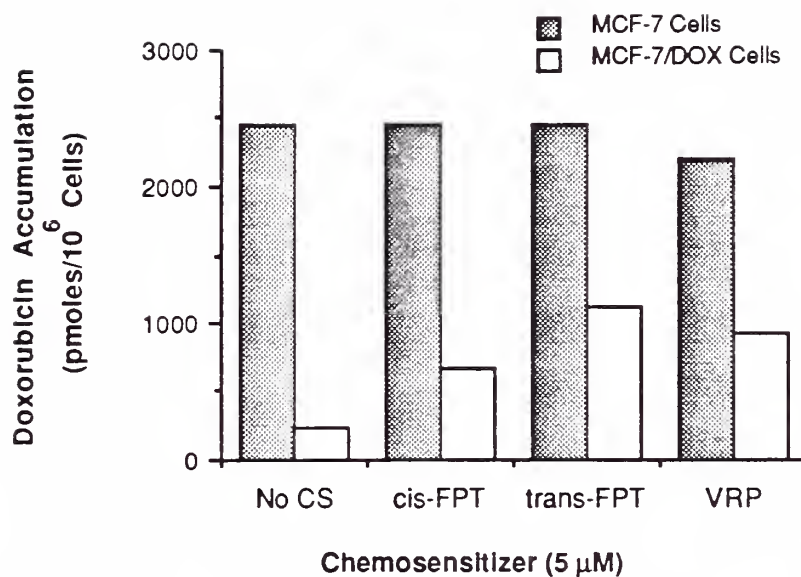




**Figure 3-1.** Effect of thioxanthene isomers on the accumulation of doxorubicin in sensitive MCF-7 (*open symbols*) and MDR MCF-7/DOX cells (*closed symbols*). Cells were incubated with 10  $\mu$ M doxorubicin in the absence (*circles*) or presence of 3  $\mu$ M *cis*-flupenthixol (*triangles*) or 6  $\mu$ M *trans*-flupenthixol (*squares*), equitoxic concentrations that alone produced 10% inhibition of cell growth. Cell associated doxorubicin at various times after the addition of drug was determined spectrofluorometrically as described in Materials and Methods. Values are means from triplicate determinations, with standard error bars when greater than 5% of the mean.

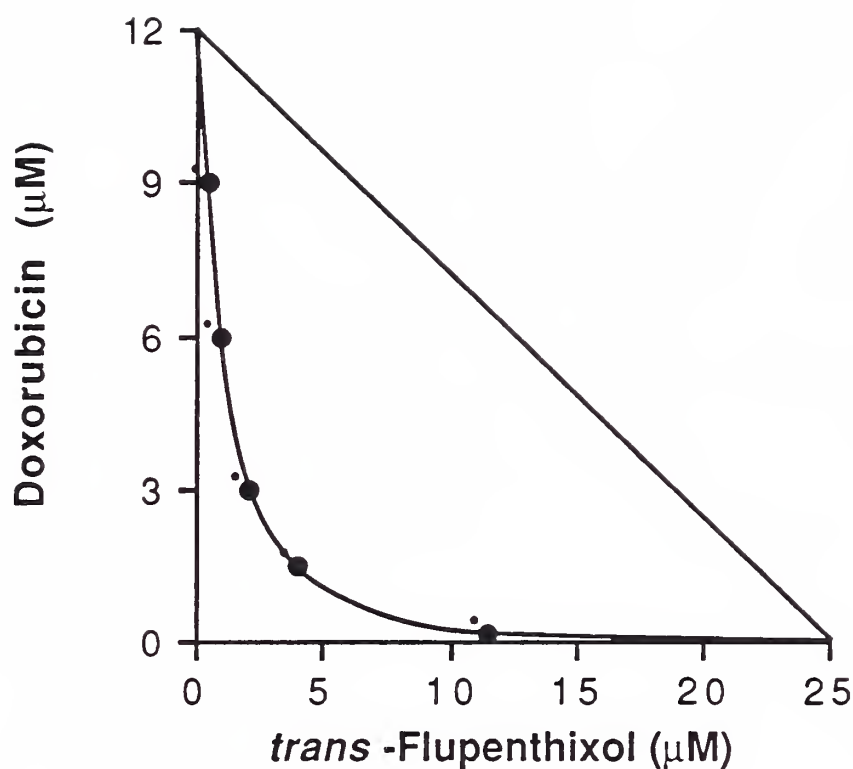






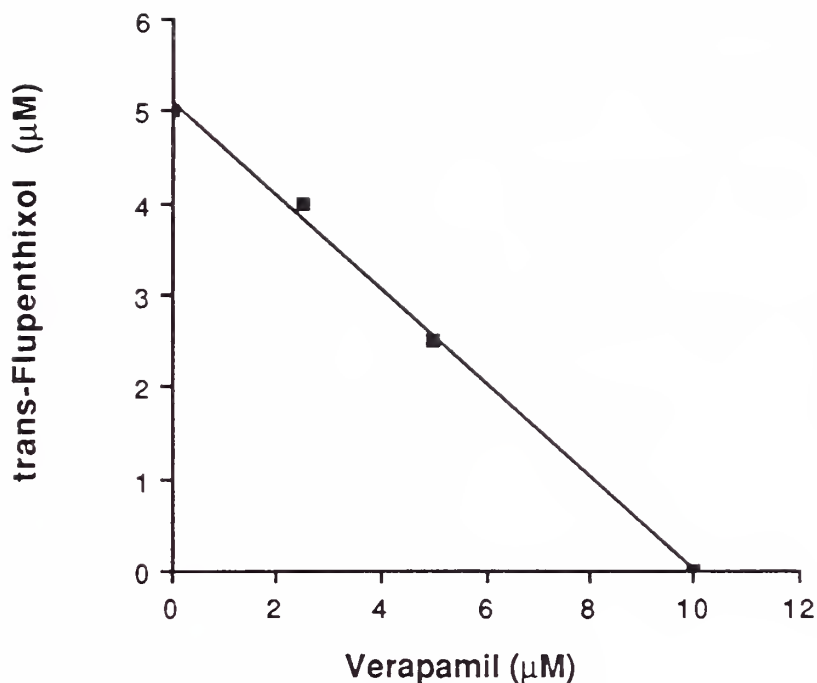
**Figure 3-2.** Effect of equimolar concentrations of chemosensitizers on the accumulation of doxorubicin in sensitive MCF-7 and MDR MCF-7/DOX cells. Cells were incubated for 3 hours with 10 μM doxorubicin in the absence (No CS) or presence of 5 μM *cis*-flupenthixol (*cis*-FPT), *trans*-flupenthixol (*trans*-FPT) or verapamil (VRP). Cell associated doxorubicin (pmoles/10<sup>6</sup> cells) was determined spectrofluorometrically as described in Materials and Methods. Values are means from duplicate determinations.





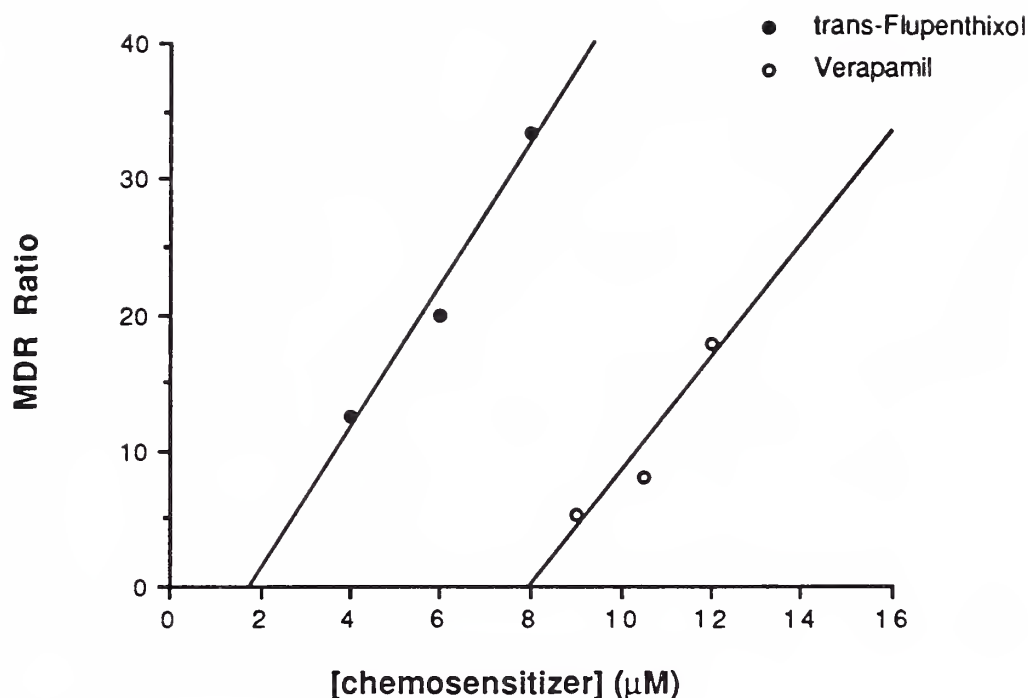
**Figure 3-3.** Isobologram analysis of the interaction between doxorubicin and *trans*-flupenthixol.  $IC_{50}$  isobole for inhibition of MCF-7/DOX cell growth by various combinations of doxorubicin and *trans*-flupenthixol (points) was determined by exposing cells to drug combinations for 48 hours as described in Materials and Methods. The straightline represents the predicted  $IC_{50}$  isobole for drugs which have additive antiproliferative effects. 12  $\mu$ M doxorubicin and 25  $\mu$ M *trans*-flupenthixol alone caused 50% inhibition of cell growth. Each point represents the mean value of quadruplicate determinations which differed by less than 5%.





**Figure 3-4.** MDR isobologram analysis of the chemosensitizing interaction between verapamil and *trans*-flupenthixol. MDR isobole for 15-fold reversal of MCF-7/DOX doxorubicin resistance by various combinations of verapamil and *trans*-flupenthixol (*squares*) was determined by exposing cells to 0 - 100  $\mu\text{M}$  doxorubicin plus combinations of chemosensitizers for 48 hours and identifying those combinations that together caused an MDR Ratio = 15. The straight line represents the predicted MDR isobole for modifiers which have additive chemosensitizing effects. 10  $\mu\text{M}$  verapamil and 5  $\mu\text{M}$  *trans*-flupenthixol alone caused an MDR Ratio = 15. Each point represents the mean value of quadruplicate determinations which differed by less than 5%.





**Figure 3-5.** Potency of chemosensitizing activity for *trans*-flupenthixol and verapamil in MDR cells. MCF-7/DOX cells were exposed to 0 - 100 μM doxorubicin for 48 hours in the absence versus presence of various concentrations of each chemosensitizer. Inhibition of cell growth and MDR Ratios were determined as described in Materials and Methods. Each point represents the fold reversal of MDR caused by that concentration of chemosensitizer, and was the result of quadruplicate determinations, which differed by less than 5%.





## Chapter IV

### *IN VIVO* EFFECT OF *TRANS*-FLUPENTHIXOL ON MULTIDRUG RESISTANCE

#### A. Introduction

The results presented in Chapters II and III demonstrate that *trans*-flupenthixol is a potent chemosensitizer in MDR cell lines *in vitro*, equal or more effective than verapamil, and much more effective than any of the previously or currently studied PTZs. While verapamil has been shown by several investigators to potentiate the effects of both doxorubicin and vinblastine *in vivo* in MDR mouse tumors (Table 1-5), the potent vasoactive effects of this drug have severely limited its potential anti-MDR use in clinical trials in humans (165). Similarly, the use of the active antipsychotic drug trifluoperazine as a chemosensitizer in humans has been limited by CNS side effects associated with its neuroleptic activity (88, 149). Conversely, *trans*-flupenthixol has been shown to be inactive compared to its *cis*-isomer as an antipsychotic agent in humans, and has not been associated with any notable side effects at clinically tested doses (116). In fact, in *in vivo* mouse studies, *trans*-flupenthixol has been shown to be 100- to 1000-fold less potent in a number of standard assays used to measure the side effects and neuroleptic potential of drugs, such as inhibition of spontaneous motor activity, cataleptic reaction, ptosis-inducing effects, and inhibition of apomorphine and amphetamine induced stereotypy (157, 158). In addition, *in vitro* studies demonstrate that [<sup>3</sup>H]-*trans*-flupenthixol completely lacks specific dopamine receptor binding activity (104), in contrast to the highly specific, potent dopamine D1 and D2 receptor binding activity displayed by [<sup>3</sup>H]-*cis*-flupenthixol (103). Also, *trans*-flupenthixol has little or no  $\alpha$ -adrenergic,  $\beta$ -adrenergic or 5-HT<sub>1</sub> blocking activity (104).

Therefore, the preclinical and clinical data reported for *trans*-flupenthixol suggest it may be an excellent candidate for the *in vivo* clinical modulation of MDR. To begin



studying the *in vivo* activity of *trans*-flupenthixol as a chemosensitizer, the P388 murine ascites tumor model was chosen. Using P388 and P388/DOX-tumor bearing mice, the toxicity of *trans*-flupenthixol and its effect on survival and cellular drug accumulation alone and in combination with doxorubicin was studied.

## **B. Materials and Methods**

### **1. Drugs**

Doxorubicin was obtained from Sigma. *Trans*-flupenthixol was generously supplied by Dr. John Hyttel (H. Lundbeck, Copenhagen, Denmark). Both drugs were freshly dissolved in distilled water immediately before each use.

### **2. Tumor Cells**

P388 sensitive and P388/DOX cells (from Dr. Ram Ganapathi) were maintained in exponential growth in suspension culture in 75 cm<sup>2</sup> Corning tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 10 µM 2-mercaptoethanol in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The P388/DOX cells were approximately 100-fold resistant to doxorubicin, and maintained a stable MDR phenotype while grown in drug-free medium for periods of up to 3 months, after which they were discarded. Cell lines were routinely tested and found to be free of contamination by mycoplasma or fungi.

### **3. Toxicity of *Trans*-Flupenthixol in Mice**

To determine the chronic toxicity of daily i.p. *trans*-flupenthixol administration, 5 to 6 week old female Swiss Webster Mice (Jackson Laboratories, Bar Harbor, Maine) weighing 18 - 22 mg were divided into 4 groups of 5 mice each. *Trans*-flupenthixol was dissolved in small amounts of distilled water and diluted with physiologic saline. Drug was injected i.p. daily for 7 days at doses of 50 to 200 mg/kg in 0.1 ml volumes. Mice were weighed daily, food and water consumption and urine and stool production noted, and mortality data collected daily. LD<sub>50</sub>'s were determined by the method of Litchfield and Wilcoxon (139).



#### 4. *Evaluation of Antitumor Activity*

Exponentially growing P388 and P388/DOX cells were washed once in PBS, and  $1 \times 10^6$  cells in 0.1 ml PBS inoculated i.p. into 8 to 10 week old female DBA/2J mice (Jackson Laboratories), weighing 19 - 23 gm each. Mice were divided into groups of 5 each, and drugs were administered daily for 10 days, starting 1 day after tumor implantation, by the method of Tsuruo (227). Accordingly, *trans*-flupenthixol (50 mg/kg) and/or doxorubicin (1 - 3 mg/kg), both dissolved in saline, were injected i.p. into mice in 0.1 ml volumes, with *trans*-flupenthixol treatment preceding doxorubicin. Response to therapy was evaluated by measuring the mean survival time for each group of mice and determining the percent increase in life span (%ILS).

#### 5. *Pharmacokinetic Studies*

To determine the accumulation of doxorubicin and *trans*-flupenthixol in ascites tumor cells *in vivo*, 2 groups of 5 DBA/2J mice each were inoculated i.p. with  $1 \times 10^6$  P388 sensitive or P388/DOX cells. Ascites cells were allowed to grow without drug treatment for 8 days, at which time tumor bearing mice were injected i.p. with 50 mg/kg *trans*-flupenthixol and/or 1 - 3 mg/kg doxorubicin in 0.1 ml saline. One hour after drug treatment, mice were etherized, and sacrificed by cervical dislocation. Ascites was removed by i.p. injection and withdrawal of 3 ml PBS. Cellular drug levels were determined by methods similar to those described in Chapter III. Briefly, cells were kept on ice and rapidly washed two times in cold PBS by centrifugation at  $100 \times g$ . Cells were resuspended in PBS, counted electronically (Coulter, Hialeh, FL), and again pelleted by centrifugation. Cells were resuspended in 2 ml of 0.3 N HCl in 50% ethanol, and sonicated for 10 pulses at 200 watt seconds with a Tekmar cell sonicator, followed by centrifugation for 60 sec at  $11,000 \times g$  using an Eppendorf 5415 microcentrifuge. Cell supernatants were removed and assayed spectrofluorometrically for doxorubicin using excitation and emission wavelengths



of 475 nm and 585 nm, respectively. Blank, control values were determined by similarly assaying ascites cell extracts from a mouse which did not receive drug treatment.

## C. Results

### 1. Toxicity of *Trans-Flupenthixol*

The toxic effects of chronic *trans*-flupenthixol administration to mice were determined for a one week period. Figure 4-1 shows the effects of 50 to 100 mg/kg daily i.p. *trans*-flupenthixol on the survival of mice. None of these dosages proved to be acutely toxic, with 100% survival after the first day of treatment. Doses greater than 50 mg/kg were chronically toxic, causing death of all mice by 7 days of daily treatment. Doses of 50 mg/kg *trans*-flupenthixol daily for 7 days was not toxic to mice, as assessed by survival (100%) and weight loss (0 %). 100 to 200 mg/kg *trans*-flupenthixol also caused sedation, diarrhea, and loss of smoothness of hair.

Since no mice treated with 50 mg/kg *trans*-flupenthixol died after 7 days, in contrast to 100% of mice treated with the higher doses tested, the LD<sub>50</sub> for this 7 day dosing regimen is difficult to determine. (The LD<sub>50</sub> after a 3 day treatment was 130 mg/kg, for instance). However, a dose of 50 mg/kg for 7 days appeared to be the maximally tolerated dose in Figure 4-1.

### 2. *In Vivo* Antiproliferative Effect of *Trans-Flupenthixol* and *Doxorubicin*

The effects of a 10 day course of 50 mg/kg *trans*-flupenthixol and 1 - 3 mg/kg doxorubicin individually, or in combination, on survival in sensitive or MDR P388-tumor bearing mice is shown in Table 4-1. Treatment with 1 mg/kg doxorubicin caused a 34% ILS in the doxorubicin-sensitive P388-tumor bearing mice, while 1 or 3 mg/kg doxorubicin did not significantly alter the median life span of P388/DOX-tumor bearing mice compared to control, untreated mice.

However, doses of 50 mg/kg *trans*-flupenthixol alone proved toxic to both groups of





tumor bearing mice, causing a 20% decrease in average length of survival. Furthermore, *trans*-flupenthixol plus doxorubicin displayed significant combined toxicity, regardless of tumor type, causing a 31 to 42% decreased survival time in mice than untreated controls.

### 3. Pharmacokinetic Studies

To determine if the doses of chemosensitizer used in this study were able to reverse the doxorubicin accumulation defect of MDR cells *in vivo*, cellular doxorubicin levels were determined for ascites cells in mice one hour after treatment with *trans*-flupenthixol and/or doxorubicin. Table 4-2 shows that when treated with 1 mg/kg doxorubicin alone, P388/DOX cells accumulate approximately 8-fold less doxorubicin than the parental cells (40.4 versus 344.8 pmoles/10<sup>6</sup> cells). However, treatment of the MDR tumor bearing mice with 50 mg/kg *trans*-flupenthixol caused a 28% and 62% increase in doxorubicin accumulation for 1 mg/kg and 3 mg/kg doxorubicin, respectively. Treatment with 3 mg/kg doxorubicin plus 50 mg/kg *trans*-flupenthixol in P388/DOX-tumor bearing cells caused nearly equivalent levels of cellular doxorubicin as in the sensitive-tumor cell bearing mice given similar doses of drug (301.6 versus 328.8 pmoles/10<sup>6</sup> cells). *Trans*-flupenthixol did not cause an increase in doxorubicin accumulation in P388 sensitive cells.

### D. Discussion

This chapter presents preliminary results from *in vivo* studies designed to determine the toxicity, appropriate dosage and scheduling, and possible anti-MDR activity of *trans*-flupenthixol. It was first determined that daily i.p. treatment of healthy mice for one week with *trans*-flupenthixol was toxic for doses  $\geq 100$  mg/kg, but caused no deaths in animals which received 50 mg/kg (Figure 4-1). The only previous toxicological testing of i.p. *trans*-flupenthixol in mice found an LD<sub>50</sub> for acute toxicity from single i.p. injections of  $270 \pm 32$  mg/kg,<sup>1</sup> while the LD<sub>50</sub> (i.p.) for racemic flupenthixol was reported as 150 mg/kg (237).

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<sup>1</sup>Hyttel, J., personal communication.



While 50 mg/kg daily i.p. *trans*-flupenthixol was well tolerated in healthy mice, for tumor bearing mice this dose proved to be toxic, causing a 20% decrease in survival. Furthermore 50 mg/kg *trans*-flupenthixol in combination with otherwise non-toxic doses doxorubicin showed greatly enhanced toxicity in tumor bearing mice (Table 4-2) than either drug alone, or no treatment.

Unfortunately, due to the intrinsic toxicity of *trans*-flupenthixol, and to the combined toxicity of *trans*-flupenthixol plus doxorubicin at the doses tested, the possible anti-tumor effects of chemosensitizing therapy with *trans*-flupenthixol could not be determined from the present results. Additional experiments are therefore in progress with changes in the dosage ( $\leq 25$  mg/kg) and route of administration (i.v., and subcutaneous, slow release pellets) of *trans*-flupenthixol treatment, to hopefully reduce toxicity and increase available concentrations of drug.

Encouraging results came from pharmacokinetic studies of the alteration in doxorubicin accumulation caused by *trans*-flupenthixol in ascites tumor cells *in vivo*, however. Specifically, while MDR P388/DOX cells accumulated nearly 10-fold less doxorubicin than sensitive P388 cells, a single i.p. dose of 50 mg/kg *trans*-flupenthixol was clearly sufficient to cause up to a 2-fold increase in doxorubicin accumulation in MDR cells, *in vivo*. Similar to *in vitro* accumulation studies, *trans*-flupenthixol did not cause an increase in doxorubicin accumulation in the sensitive cells, *in vivo*. Thus, while the presently studied doses of *trans*-flupenthixol and doxorubicin demonstrated systemic toxicity in mice, they also functioned to partially reverse the accumulation defect in MDR cells *in vivo*. Hopefully, reducing the dose of *trans*-flupenthixol will eliminate toxicity but still cause increased doxorubicin accumulation in MDR cells *in vivo*, resulting in an increased %ILS.

As discussed in Chapter I, there are a number of other mouse tumor models which may more realistically reflect clinical MDR tumors in humans. If significant chemosensitizing activity is shown for *trans*-flupenthixol in combination with doxorubicin in the P388/DOX screening model, further studies of the effect of *trans*-flupenthixol plus various cytotoxic



drugs or drug combinations on solid tumor growth and drug pharmacokinetics in nude mice with transplanted human tumor xenografts such as MCF-7/DOX, or an *mdr1* transfected tumor cell line, may provide interesting and clinically useful data.

While the results from this pilot *in vivo* murine trial were disappointing due to the unexpected toxicities, there is evidence to suggest that *trans*-flupenthixol may have great potential for clinical use as a chemosensitizer. As discussed in Chapter II, clinical trials of the antipsychotic effects of thioxanthene isomers have shown *trans*-flupenthixol to completely lack dopaminergic activity and extrapyramidal side effects (116). Furthermore, sensitive chromatographic and radioimmunoassays have been developed for the measurement of tissue and plasma *cis*- and *trans*-flupenthixol levels for studies of the psychopharmacologic effects of thioxanthenes in humans (117, 170, 194) which have generated important clinical pharmacokinetics data possibly relevant to their proposed use as modulators of tumor resistance. For example, flupenthixol has been shown to undergo extensive first-pass hepatic metabolism and to have a high systemic clearance due to its high hepatic extraction ratio, with mean systemic availability ranging from 30 - 70% (118). Nevertheless, flupenthixol possesses an apparent half-life of elimination of 34 to 36 hours due to a large volume of distribution (20 L/kg) (119). Also, flupenthixol's lipophilicity results in selective partitioning into tissues (suggesting high tumor tissue levels may be possible), making correlations between plasma levels and clinical effects notoriously difficult (10). Reported peak plasma levels for *cis*-flupenthixol treatment (1 mg i.v. or 4 mg p.o.) have been 3 - 4  $\mu\text{g/L}$  ( $\sim 7$  nmolar) (120), 1000-fold less than concentrations necessary for *in vitro* chemosensitizing activity. However, it is possible that far greater doses of *trans*-flupenthixol may be safely administered to humans, due to its apparent lack of clinical activity. In addition, a recent report measuring the steady state levels of *cis*- and *trans*-flupenthixol resulting from 4 to 15 mg daily po doses of a 1:1 mixture of both drugs resulted in *trans*-flupenthixol levels of up to 20 nmolar, and indicated that 2-fold greater *trans*-flupenthixol steady state plasma concentrations were achieved in comparison to *cis*-flupenthixol (11). These data suggest that it may be possible to achieve sufficient tissue



levels of *trans*-flupenthixol capable of producing anti-MDR activity in human tumors.

## E. Summary and Conclusions

This thesis has attempted to better define a structural 'pharmacophore' for the reversal of MDR, and characterized the *in vitro* and *in vivo* chemosensitizing activity of a newly described class of anti-MDR agents, and has begun to explore the mechanisms involved in the pharmacological reversal of MDR.

Specifically, systematic structure-activity relationships for the PTZs' ability to reverse MDR in a human breast cancer cell line revealed the specific structural features, such as the length and type of amino side chain, that in addition to hydrophobicity determined the chemosensitizing activity of these drugs. Based on these predictions, the thioxanthene class of chemosensitizers was identified, and shown to be potent and stereospecific antagonists of MDR. The thioxanthenes *cis*- and *trans*-flupenthixol were further characterized for chemosensitizing activity in several *in vitro* and *in vivo* MDR tumor cell lines, and shown to partially reverse MDR and cause increased cytotoxic drug accumulation in cells which overexpress P-gp. Furthermore, the mechanism of chemosensitizing effects of PTZs and thioxanthenes was shown unlikely to be due to their known inhibition of CaM and PKC.

The results presented in this thesis have a number of implications for future basic and clinical research in the field of cancer drug resistance. First, based on the structural principles derived from the PTZ anti-MDR model, it may be possible to rationally design and synthesize novel drugs which possess greater chemosensitizing activity and less toxic side effects than those currently known. Also, the identification of thioxanthenes as potent, stereospecific chemosensitizers may provide useful pharmacologic probes for exploring the biochemical basis of MDR and its reversal. Finally, the preliminary *in vivo* anti-MDR data presented, together with the interesting clinical and pharmacological profile already known for *trans*-flupenthixol, suggest this drug may be a potentially useful candidate for clinical trials testing the ability of drugs to modulate tumors resistance in humans.

In conclusion, a great deal of information has been gathered in the last decade





concerning the cellular pharmacology and molecular biology of MDR cell lines *in vitro*, and an understanding of the relevance and mechanism of MDR and P-gp in human tissues and tumors is now beginning. The discovery of the chemosensitizer *trans*-flupenthixol may hopefully assist to further advance the knowledge of the mechanisms of MDR and its reversal, and potentially contribute to the clinical reversal of tumor resistance in humans.



TABLE 4-1

*In Vivo* Chemosensitizing Activity of *trans*-Flupenthixol

Tumor Drugs and Dose (mg/kg)	Survival	
	Days	% ILS
P388/S		
Control	13.0 $\pm$ 0.7	0
DOX (1)	17.4 $\pm$ 0.9	+34
tFPT (50)	10.4 $\pm$ 2.1	- 20
DOX (1) + tFPT (50)	7.6 $\pm$ 1.5	- 42
P388/DOX		
Control	10.4 $\pm$ 1.3	0
DOX (1)	10.2 $\pm$ 0.8	- 2
DOX (3)	9.8 $\pm$ 0.4	- 6
tFPT (50)	8.2 $\pm$ 2.5	- 21
DOX (1) + tFPT (50)	6.4 $\pm$ 2.2	- 38
DOX (3) + tFPT (50)	7.0 $\pm$ 0.7	- 31

Mean survival time in days ( $\pm$  S.E.) and percent increase in life span (%ILS) for P388/S and P388/DOX-tumor bearing mice (5 mice per condition).  $1 \times 10^6$  sensitive or MDR cells were injected i.p. in adult, female DBA/2J mice on day 0, and drugs given daily from Day 1 to Day 10 dissolved in 0.1 ml saline.



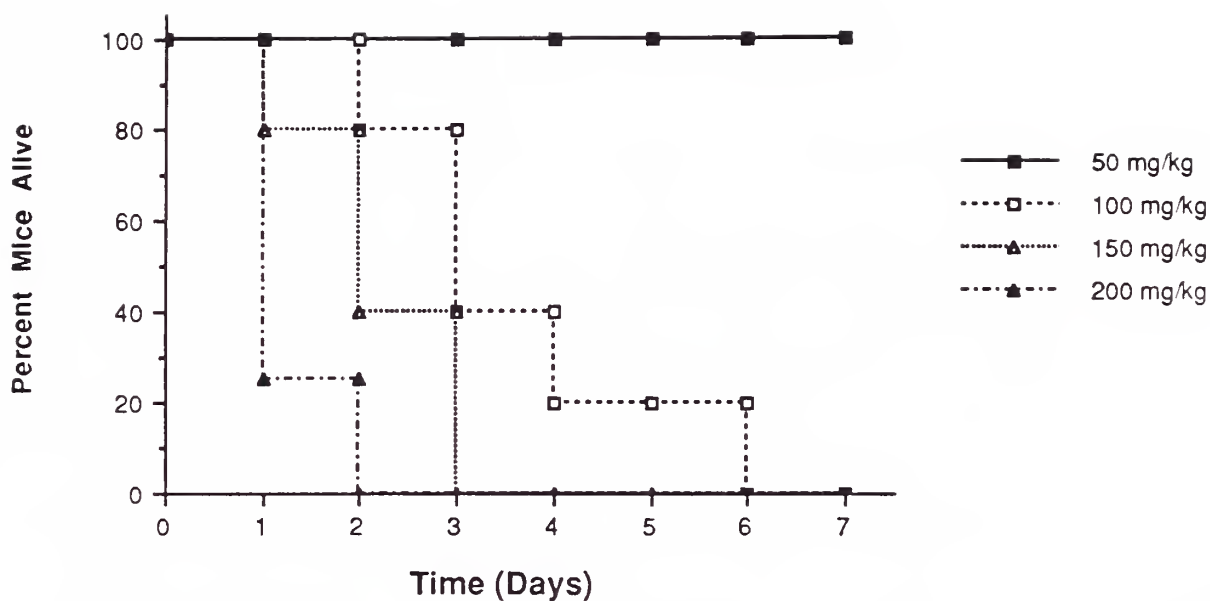
TABLE 4-2

Effect of *trans*-Flupenthixol on *In Vivo* Doxorubicin Accumulation  
in Sensitive and MDR Tumor Bearing Mice

Drugs and Dose (mg/kg)	<u>Doxorubicin (pmoles/10<sup>6</sup> cells)</u>	
	P388	P388/DOX
tFPT (50)	0.0	0.0
DOX (1)	344.8	40.4
DOX (3)	ND	185.8
DOX (1) + tFPT (50)	296.2	51.8
DOX (3) + tFPT (50)	328.8	301.6

DBA/2J mice were injected i.p. with  $1 \times 10^6$  P388 or P388/DOX cells, and received no treatment for 8 days. Drugs were injected i.p. in 0.1 ml saline one hour before ascites cells were removed and assayed spectrofluometrically for doxorubicin accumulation (pmoles/10<sup>6</sup> cells) as described in Materials and Methods. Control ascites cell levels were from a mouse that did not receive treatment.





**Figure 4-1.** Toxicity of *trans*-flupenthixol in mice. Adult, female Swiss Webster mice were divided into 4 groups of 5 mice each and various doses of drug in 0.1 ml saline were injected i.p.daily for 7 days. Surviving animals were observed daily for 2 weeks, and mortality expressed as a percentage of total mice per group.





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